# Functions of the Mineralocorticoid Receptor in the Hippocampus

by

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# Table of Contents

# List of Figures

# List of Tables

Abstract

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In the central nervous system, glucocorticoids influence neuroendocrine function, cognition, neurogenesis, neurodegeneration, and cell survival. Glucocorticoid hormones signal through the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR), closely related members of the steroid hormone receptor superfamily. Their expression profiles and modes of action suggest both overlapping and distinct functions in mediating glucocorticoid effects. As GR function has been widely examined, research in this thesis focuses on the roles of MR action in the central nervous system using both *in vivo* and *in vitro* approaches. First, we generated a transgenic mouse model that overexpresses MR in the forebrain (MRov). Relative to wild-type littermate controls, MRov mice display reduced anxiety-like behaviors and exhibit suppressed HPA axis activity in response to stress. These data demonstrate that functions of forebrain MR can both overlap (regulation of neuroendocrine function) and oppose (modulation of anxiety-like behavior)

GR-mediated actions. Second, we utilized the mouse hippocampal cell line, HT-22, to address corticosteroid receptor-mediated effects on both cell survival and regulation of transcription in an *in vitro* system. HT-22 cells express GR but not MR, and have been shown to be sensitive to glutamate toxicity in a manner that is exacerbated by activation of GR. To address the role of MR in this "glucocorticoid endangerment", we generated stable clones of HT-22 cells that express MR in addition to GR. Using these cell lines, we confirmed that while GR activation enhanced glutamate toxicity, the co-activation of MR and GR in the HT-22/MR clone attenuated the glucocorticoid endangerment of the cells. Finally, MR- and GR-mediated regulation of glucocorticoid responsive genes was monitored at the transcriptome level in HT-22/Parent and HT-22/MR cells. This research demonstrated that co-activation of MR and GR resulted in the regulation of a substantially larger set of genes relative to GR activation alone, including classes of genes known to regulate cell survival and proliferation, suggesting that changes in the balance of receptor levels may result in functionally significant alterations in global transcriptome regulation. Together, these data reveal important overlapping and distinct roles for hippocampal MR and GR.

# Chapter 1

## Introduction

When an organism is confronted with a situation that is perceived as threatening, a myriad of events occur that prepare the organism for a response that is typically characterized as "fight-or-flight". Physiological manifestations of this fight-or-flight response that are common to all mammals include but are not limited to, increased heart rate, increased respiration, and increased vigilance. The body also mobilizes energy sources and redirects the use of that energy to areas that most need it. These efforts are energetically costly but serve one very important purpose, the maintenance of an internal equilibrium that allows the organism to survive in a constantly changing environment.

This concept was formally proposed by Claude Bernard who noted in his 1865 work, *Introduction to Experimental Medicine*, "Constance of the internal environment is the condition for a free and independent life" (Bernard 1865). It was over half a century later, however, before Walter Cannon used the term "homeostasis" to describe the "fundamental condition of stability" of an organism, and the ability of "various physiological arrangements which serve to restore the normal state when it has been disturbed" (Cannon 1932). Cannon was the first to describe the acute changes in secretions from the adrenal gland that were associated with what he called the fight-or-flight response. He found that any number of a variety of threats to homeostasis, such as hypoglycemia, cold exposure, or emotional distress resulted in the activation of both the

adrenal medulla and the sympathetic nervous system, or the "sympathoadrenal" system that was thought to function as a single unit. Cannon also proposed that adrenaline was the factor that was both released by the adrenal medulla and which served as the neurotransmitter of the sympathetic nervous system, although it was later determined that noradrenaline actually served as the primary neurotransmitter of the sympathetic nervous system (Goldstein and Kopin 2007). It was thought that deviations from normal parameters were brought back in line automatically by local negative feedback mechanisms in each organ.

While earlier researchers paved the way with the concepts of homeostasis, Hans Selye was instrumental in formalizing the concept of "stress". Selye defined stress as "the nonspecific response of the body to any demand upon it" (Selye 1936). His research suggested that an organism exhibited three universal stages of coping with exposure to stress, or the "General Adaptation Syndrome". Selye termed the first stage of this syndrome the "general alarm reaction", analogous to Walter Cannon's fight-or-flight response. The second stage was characterized as a period of adaptation where the organism showed resistance to the stressor. Finally, if the stress was of sufficient intensity and occurred over a long period of time, a third stage would be reached that was characterized as a period of exhaustion and eventual death. Selye's work also emphasized the activity of another body system in the general stress response, the hypothalamic-pituitary-adrenal (HPA) axis. It was later demonstrated that steroid hormones released from the adrenal gland during stress were capable of participating in both the resistance to the stressor and eventually to the pathological states resulting from extended exposure to stress, concepts that are key to the research of this thesis.

The works of both Cannon and Selye emphasized one concept that has more recently been challenged, the idea of the general nature of the stress response. It was originally believed that any kind of stressor would result in the same pattern of activation of the "sympathoadrenal" and HPA systems. As more intensive research ensued, however, it became clear that this was not always the case. Table 1-1 depicts data from a large number of studies indicating the relative activation levels of the HPA axis, the adrenomedullary hormone system (AHS), which releases adrenaline into the blood stream, and the sympathetic noradrenergic system (SNS) during exposure to different stressors. It is clear that different stressors elicit different response patterns from each of the three systems, and that activation of the AHS actually more closely resembles activation of the HPA axis. This last observation serves to functionally uncouple the activities of the AHS and SNS for many types of stressors.

Along with the realization that different stressors are capable of eliciting different patterns of activation, the broader realization that almost every physiological parameter changes dramatically throughout the day and in response to varying physiological and psychological demands required a new concept of "stress". In 1988, Sterling and Eyer introduced the idea of "allostasis", broadly meaning stability through change. Instead of a model dictating that the internal equilibrium is maintained by the local actions of each individual organ, the allostatic model dictates that the internal environment is always changing to meet perceived and anticipated demands. Moreover, local homeostatic control of individual organs is subordinate to the brain, the site where the effects of different stressors are realized and where appropriate behavioral and physiological changes are organized (Sterling P 1988). Importantly, the ability of an organism to adapt

**Table 1-1 Hypothalamo-pituitary-adrenocortical (HPA), adrenomedullary hormonal system (AHS), and sympathetic nervous system (SNS) responses to different stressors**

| | HPA | AHS | SNS |
|---|---|---|---|
| Cold Exposure, No Hypothermia | 0 | + | +++ |
| Active Escape/Avoidance | + | + | ++ |
| Hemorrhage, No Hypotension | + | + | ++ |
| Surgery | + | + | ++ |
| Exercise | + | ++ | +++ |
| Cold Exposure, Hypothermia | + | ++ | ++++ |
| Social Stress in Monkey | ++ | ++ | ++ |
| Laboratory Mental Challenge | ++ | ++ | + |
| Hemorrhagic Hypotension | +++ | +++ | + |
| Passive/Immobile Fear | ++ | +++ | + |
| Public Performance | ++ | +++ | + |
| Pain | ++ | +++ | ++ |
| Exercise to Exhaustion | ++ | +++ | ++++ |
| Glucoprivation | +++ | ++++ | + |
| Fainting | ++ | ++++ | 0 |
| Immobilization in Rat | ++++ | ++++ | ++++ |
| Cardiac Arrest | +++ | ++++ | ++ |

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through allostatic processes is dependent on genetic makeup, developmental history, and previous experiences.

The HPA axis plays a critical role in the ability of an organism to adapt to a homeostatic challenge. The end result of HPA axis activation is secretion of glucocorticoid hormones (corticosterone in rodents, cortisol in humans) into the bloodstream that serve to mobilize and redirect energy resources throughout the body. The physiological parameters that are altered in this transition from basal to active are largely catabolic in nature, resulting in the breakdown of metabolic compounds to produce energy. As a consequence of this biochemically catabolic state of "arousal", other physiological processes associated with anabolic processes, such as immune system function, digestion, reproduction, and wound healing are all suppressed (Sterling P 1988). While the short-term activation of these allostatic processes are highly adaptive, long-term activation may have cumulative adverse effects including osteoporosis, hypertension, and increased risk for the development of multiple mood disorders including major depression and several anxiety disorders (De Kloet, Vreugdenhil et al. 1998). This process of harm caused by allostatic processes is referred to as "allostatic load" (McEwen 2000).

The following sections will focus on both physiological and molecular aspects of the activation and regulation of the HPA axis as an allostatic system that is necessary for survival. Dysregulation of the system and subsequent pathologies associated with allostatic load derived from over-activation of the HPA axis will also be discussed with particular emphasis on mood disorders.

***HPA axis activity:***

The HPA axis operates in two distinct realms of activity, the basal unstressed state and the stressed state. Under basal resting conditions, the HPA axis is activated in a circadian fashion with low levels of circulating corticosteroids at the circadian trough (sleeping phase) and higher levels at the circadian peak (waking phase). Several studies suggest that the suprachiasmatic nucleus (SCN) regulates the circadian control as lesions of the SCN results in the disappearance of the rhythm of corticosteroid release (Moore and Eichler 1972; Abe, Kroning et al. 1979; Watanabe and Hiroshige 1981). HPA axis activity is also regulated under stress conditions; here, two primary modes of activation can be identified. Herman and colleagues distinguish between "reactive" and "anticipatory" stress responses (Herman, Figueiredo et al. 2003). "Reactive" stress responses are characterized by a homeostatic challenge that is of physiological origin. This would include abrupt changes in cardiovascular tone, hypoglycemia, or blood-borne cytokine factors signaling an infection (Table 1-2). "Anticipatory" stress responses, on the other hand, occur in the absence of a physiological challenge. Instead, the HPA axis is activated in response to a perceived threat to homeostasis, such as predator odor, or restraint (Table 1-2).

Stress regulation of the HPA axis by either the "reactive" or "anticipatory" modes occurs through activation of corticotropin releasing hormone (CRH)- and arginine vasopressin (AVP)-containing neurons located within the hypothalamic paraventricular nucleus (PVN). These neurons are stimulated to synthesize and release CRH and AVP by various signaling mechanisms generated from afferent pathways that are relayed to the PVN via different brain regions depending on the mode of activation ("reactive" vs.

**Table 1-2 Stimuli triggering "reactive" vs. "anticipatory" HPA stress responses**

| "Reactive" Responses | "Anticipatory" Responses |
|---|---|
| Pain | Innate programs |
| Visceral | Predators |
| Somatic | Unfamiliar environments/situations |
| Neuronal homeostatic signals | Social challenges |
| Chemoreceptor stimulation | Species-specific threats |
| Baroreceptor stimulation | - illuminated spaces for rodents |
| 'Osmoreceptor' stimulation | - dark spaces for humans |
| Humoral homeostatic signals | Memory programs |
| Glucose | Classically conditioned stimuli |
| Leptin | Contextually conditioned stimuli |
| Insulin | Negative reinforcement/frustration |
| Renin-angiotensin | |
| Atrial natriuretic peptide | |
| Others | |
| Humoral inflammatory signals | |
| IL-1 | |
| IL-6 | |
| TNF-$\alpha$ | |
| Others | |

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"anticipatory", Figure 1-1). Upon release, CRH travels through the hypophyseal portal system to anterior pituitary corticotropes where it increases the synthesis and release of ACTH. ACTH travels through the blood and stimulates the production and release of glucocorticoid hormones from the adrenal gland. The "reactive" pathways arising from brainstem regions signal directly to the PVN. Conversely, "anticipatory" pathways arising from various forebrain limbic structures such as the hippocampus, amygdala, and the prefrontal cortex, are thought to relay through multiple other brain regions that in turn signal to the PVN. Interestingly, some of these "anticipatory" pathways relay through regions that overlap with the "reactive" pathway of HPA axis activation (Herman, Figueiredo et al. 2003).

While the "reactive" pathway is largely excitatory for CRH release, "anticipatory" signaling from various brain limbic regions is both excitatory and inhibitory. Lesioning and stimulation studies of the hippocampus suggest that this region largely provides inhibitory input to the PVN. Lesions have been shown to promote both basal hypersecretion of corticosteroids (Fendler, Karmos et al. 1961), as well as prolong the corticosteroid response to stress (Herman, Cullinan et al. 1995). Conversely, stimulation of the hippocampus reduces HPA axis activity in both humans (Rubin, Mandell et al. 1966) and rats (Casady and Taylor 1976). The medial prefrontal cortex has also been shown to provide negative feedback to the HPA axis. Lesions of this region enhance ACTH and corticosterone responses to restraint stress but do not affect basal circadian ACTH or corticosterone levels, suggesting that regulation from this region is selective for stress-induced modulation of HPA axis activity (Diorio, Viau et al. 1993; Figueiredo, Bruestle et al. 2003). While the hippocampus and the prefrontal cortex inhibit HPA axis

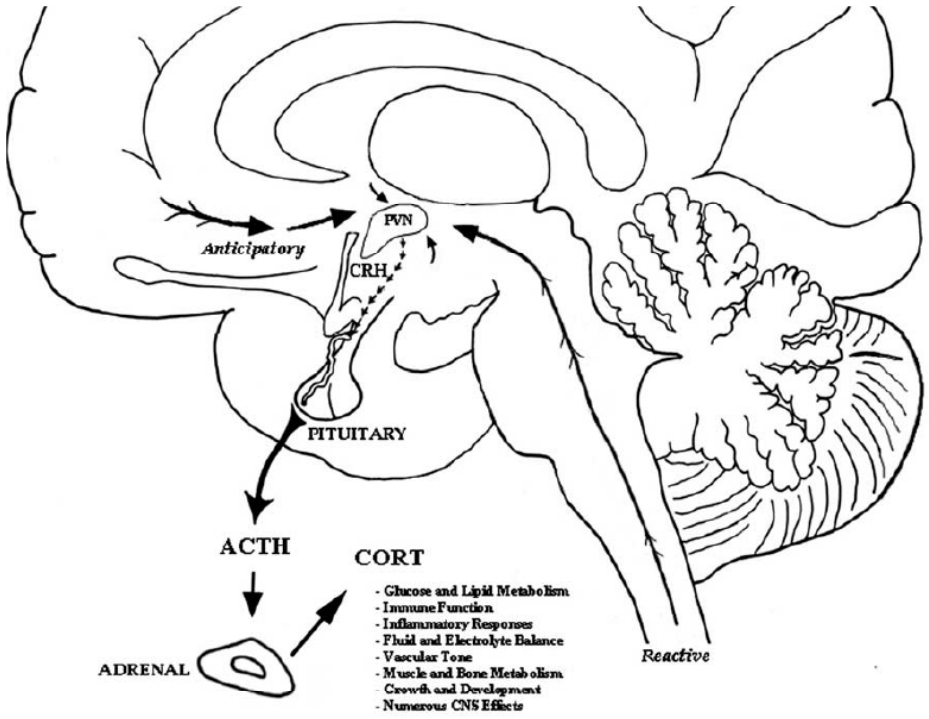


**Figure 1-1 Overview of the HPA axis, including principal classes of regulatory afferents and corticosteroid actions.**

CRH neurons located within the hypothalamic PVN drive pituitary corticotropes via the portal vasculature, stimulating the release of ACTH which, in turn, stimulates the synthesis and release of corticosteroids (Cort) from the adrenal glands. CRH neurons in the PVN are regulated by sensory afferents from the brainstem that provide largely "reactive" signals that are generally excitatory and relatively direct. Conversely, it is hypothesized that "anticipatory" signals are conveyed from forebrain limbic structures to the PVN via multisynaptic pathways and include both excitatory and inhibitory information. Reprinted from Frontiers in Neuroendocrinology 24, Herman, J.P., Figueirido H, Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenal responsiveness, 151-180, (2003), with permission from Elsevier.

activity, signaling from the amygdala appears to activate it. Electrical stimulation of the amygdala promotes corticosteroid secretion in rats (Redgate and Fahringer 1973), and lesion studies suggest that selective damage of the central nucleus of the amygdala decreases ACTH and corticosteroid release after immobilization stress (Beaulieu, Di Paolo et al. 1986).

These findings fit well with an allostatic model of stress integration for HPA axis activity. The initiation of HPA axis activity occurs through the coordinated actions of multiple pathways. As the "reactive" pathway partially overlaps with the "anticipatory" pathway that involves limbic regions concerned with both learning and memory and the integration of behavioral responses to fear and anxiety, the ultimate release of CRH from the PVN in response to any particular stressor could be experience-dependent. This mnemonic aspect of stress integration could, for example, reduce HPA responsiveness to contextual stimuli with repeated exposure, allowing the organism to habituate and reduce future HPA activation (Herman, Figueiredo et al. 2003). Thus, the allostatic model allows a more fine-tuned response to a stressor.

***HPA axis activation and mood disorders:***

Proper function of the HPA axis is necessary for survival. Glucocorticoids are important for maintenance of blood pressure and cardiovascular function; in addition, they regulate metabolism, slow the immune system's inflammatory response and help to maintain proper arousal. As all of these functions are essential for survival and for adaptation and coping with psychological and physiological stressors, dysregulation of the HPA axis forms the basis of pathology associated with a number of psychiatric

disorders including major depressive disorder (MDD), psychotic depression, bipolar disorder and several anxiety disorders (Chrousos and Gold 1992). A high percentage of patients with MDD exhibit increased corticosteroid levels indicative of a "hyperactive" HPA axis. Evidence that corticosteroid exposure by itself can have negative effects on mood is seen in patients that receive long-term treatment with corticosteroids. Changes in mood including hypomania, mania, psychosis and depression have been reported (Brown and Suppes 1998). Similarly, patients with Cushing's syndrome, who suffer from excess plasma cortisol levels, exhibit higher rates of depression that appear to be reversed upon treatment that reduces cortisol levels (Starkman, Schteingart et al. 1986).

Further evidence linking hypercortisolemia to depression comes from three classes of studies. The first studies showed that the corticosteroid synthesis inhibitor, metyrapone, proved effective in producing antidepressant-like behavior in rodent models (Healy, Harkin et al. 1999), as well as in clinical trials (O'Dwyer, Lightman et al. 1995). A second study showed that blocking glucocorticoid action proved to be efficacious in the treatment of psychotic depression (DeBattista and Belanoff 2006). Finally, multiple studies have shown that clinically efficacious treatment with antidepressants was preceded by a normalization of HPA axis activity in those depressed patients who were typified by hypercortisolemia, (Holsboer, Dorr et al. 1982; Greden, Gardner et al. 1983; Holsboer, Steiger et al. 1983). Conversely, the persistence of HPA axis dysfunction during antidepressant treatment appeared to be associated with a higher probability of relapse (Ribeiro, Tandon et al. 1993; Heuser, Schweiger et al. 1996).

While the exact mechanisms underlying the hyperactivity of the HPA axis remain unclear, both dysregulation in negative corticosteroid feedback as well as an increased

drive of the HPA axis have been suggested (Young, Kotun et al. 1993; Young, Haskett et al. 1994; Steckler, Holsboer et al. 1999). However, the pertinent question of whether increased drive of the HPA axis is the cause or the consequence of disturbed negative feedback is not known at this point. Regardless of this distinction, evidence that there is a profound dysregulation in the HPA axis comes from a neuroendocrine function test known as the combined dexamethasone/CRH challenge test. In this test, dexamethasone pretreatment is used to suppress corticosterone production and then the axis is stimulated with a dose of CRH. A high percentage of depressed patients demonstrate two features in this test: 1) a non-suppression of cortisol to dexamethasone pretreatment; and 2) a subsequent hyperactive cortisol response to the CRH treatment [see Figure 1-2 (von Bardeleben and Holsboer 1991)]. Both of these responses are consistent with disturbed glucocorticoid feedback (Modell, Yassouridis et al. 1997).

It is hypothesized that the chronic elevations in corticosteroids seen in some depressed patients can be damaging to the brain in general, and to the hippocampus in particular, possibly contributing to deleterious effects on its structure and function and to the behavioral and physiological sequelae that accompany many diseases of affect (Lee, Ogle et al. 2002). As the hippocampus is a site of negative feedback for the HPA axis, the decrease in functional hippocampal integrity results in continued elevation of corticosteroid levels and further degeneration. This glucocorticoid feed-forward cascade may be a potential mechanism to explain the degenerative process. In the following sections, I will introduce the two corticosteroid receptors and discuss their roles in HPA axis regulation and anxiety-related behavior as well as their contributing roles to hippocampal structure and function.

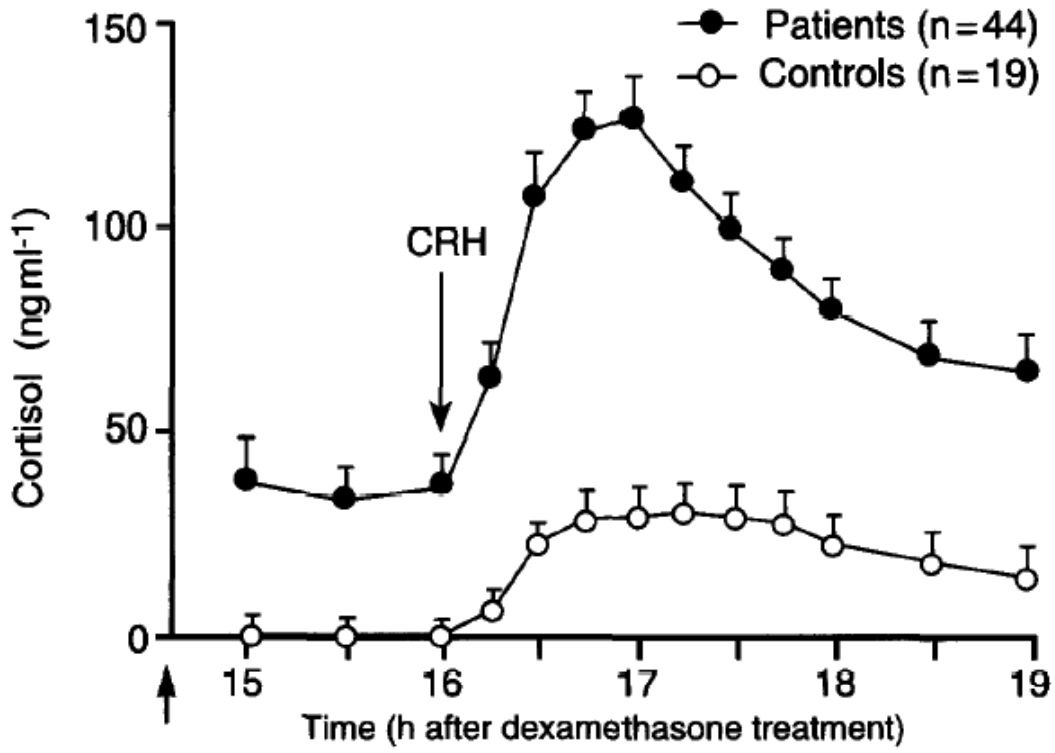


**Figure 1-2 Disturbed feedback in patients with major depressive disorder.**

After dexamethasone pretreatment (1.5mg), plasma concentrations of cortisol were increased the next day among patients with major depression. Upon stimulation with CRH (100μg at 1600hours after dexamethasone), patients responded with much higher plasma cortisol levels relative to controls suggesting disturbed feedback regulation of the HPA axis in depression. Reprinted from Biological Psychiatry 29(10), Bardeleben, U., and Holsboer, F., Effect of age on the cortisol response to human corticotropin-releasing hormone in depressed patients pretreated with dexamethasone, 1042-1050, (1991), with permission from Elsevier.

### *Corticosteroid Receptors: Mineralocorticoid Receptor (MR) and Glucocorticoid Receptor (GR)*

A vast array of developmental, physiological, and behavioral processes are controlled by steroid hormones. The actions of these hormones are mediated by an evolutionarily conserved family of intracellular receptors that function as ligand-activated transcription factors to coordinate the expression of gene networks (Evans 1988). Sequence homology studies reveal that a prominent feature of members of this family is their organization into three main functional domains: 1) an N-terminal domain that contains sequences important for transcriptional regulation; 2) a central region containing sequences necessary for receptor/DNA interactions; and 3) a carboxy-terminal ligand-binding domain required for receptor/hormone interactions and transcriptional regulation.

The actions of corticosteroid hormones in the brain are mediated by two of these receptors, the type I mineralocorticoid receptor (MR) and the type II glucocorticoid receptor (GR). MR and GR are two of the most closely related members of this family of hormone receptors and as such, they share several features in common. The ligand binding domain of MR and GR share 57% amino acid identity, consistent with their ability to bind a similar group of glucocorticoid hormones. However, MR can bind many corticosteroids with an affinity that is roughly 10-fold greater than GR (0.5nM vs. 5nM, respectively for corticosterone). MR also binds aldosterone with a similar affinity as corticosterone, but GR is largely insensitive to aldosterone at physiological concentrations.

The DNA binding domains of MR and GR allow them to bind to sequences of DNA known as glucocorticoid responsive elements (GRE: GGTACAnnnTGTTCT)

found in the promoter regions of corticosteroid responsive genes. MR and GR are highly homologous in the DNA-binding domain, sharing an amino acid identity of 94%, which results in their ability to bind to the same GREs. The N-terminal regions of MR and GR are the most divergent, sharing only 15% amino acid identity. The functional implications of the diversity in this region will be further discussed in the Mechanisms of Corticosteroid Receptor-Mediated Gene Expression section.

When not bound by hormone, MR and GR normally reside in the cytoplasm and are part of a multi-protein complex anchored by heat shock protein-90 (HSP90), but also comprised of other heat shock proteins and immunophilins that serve to hold the receptor in a conformation that facilitates hormone binding (Pratt, Morishima et al. 2006). Once the hormone is bound, a conformational change is induced that allows the chaperone complex to dissociate from the receptor, exposing nuclear localization signals that direct translocation of the receptors to the nucleus where they exert transcriptional control over glucocorticoid responsive genes

However, it should be noted that evidence exists for a membrane bound corticosteroid receptor capable of mediating cellular actions via a non-genomic mechanism. In the roughskin newt (*Taricha granulosa*), a putative membrane GR was isolated in neuronal membranes that has characteristics of a G-protein coupled receptor (Orchinik, Murray et al. 1991). Ligand binding studies have been used to characterize putative membrane corticosteroid receptors in the rat liver (Allera and Wildt 1992), rat brain (Sze and Towle 1993), and the pituitary cell line AtT-20 (Harrison, Balasubramanian et al. 1979), however, the membrane glucocorticoid receptor has not yet been cloned or fully characterized.

MR and GR exhibit distinct expression profiles in rodents and primates. GR is widely expressed throughout the periphery and the brain, whereas the expression pattern of MR is much more limited. In the periphery, MR is most highly expressed in the epithelial target tissues of the kidney and the colon, as well as non-epithelial tissues such as cardiomyocytes, the blood vessel wall, and circulating monocytes (Fuller and Young 2005). In epithelial target tissues such as the kidney, MR functions as a regulator of sodium balance by binding aldosterone that is released from the adrenal in response to multiple factors including changes in plasma $K^+$ and $Na^+$ concentrations. Aldosterone concentrations in the plasma are roughly 100-1000 fold lower than corticosterone/cortisol concentrations, but aldosterone specificity is maintained in $Na^+$-transporting epithelial cells through expression of the enzyme 11 beta-hydroxysteroid dehydrogenase II (11βHSD-II) that converts corticosterone/cortisol to the inactive cortisone. In the central nervous system, the expression of 11βHSD-II is restricted to the anterior hypothalamus and circumventricular organs; no detectable amounts of 11βHSD-II are found in the hippocampus (Roland, Krozowski et al. 1995).

In the central nervous system of rodents, MR is detected in the prefrontal cortex, the medial and central amygdala, lateral septum, various thalamic nuclei and several hypothalamic nuclei, but the highest expression levels of MR are found in regions CA1, CA2, CA3 and the dentate gyrus of the hippocampus (Herman, Patel et al. 1989; Ahima, Krozowski et al. 1991; Han, Ozawa et al. 2005). Figure 1-3 is an *in situ* hybridization study that depicts the anatomical overlap of MR and GR mRNA in the mouse brain. It is clear from these studies that the highest levels of co-expression of MR and GR are contained in the hippocampus. In contrast, MR is expressed at high levels, not only

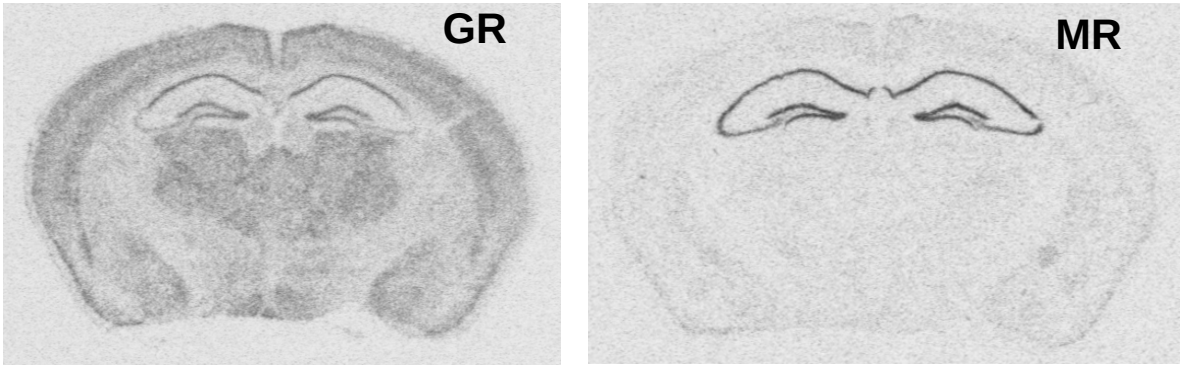


**Figure 1-3 Coronal sections of mouse forebrain depicting anatomical distribution of GR and MR mRNA.**

*In situ* hybridization with $^{35}S$ labeled GR and MR specific cRNA probes.

in the hippocampus, but also in cerebral cortex in humans and squirrel monkeys, suggesting additional roles for cortical MR in primates (Patel, Lopez et al. 2000).

MR and GR expression are both subject to regulation by glucocorticoids, an effect that predominantly occurs through activation of MR. Adrenalectomy (ADX) results in rapid increases in MR protein within 12 hours and is reversed by replacement with MR selective but not GR selective agonists (Kalman and Spencer 2002); treatment of ADX rats with an MR selective agonist also prevents the ADX-induced increase in GR binding (Miller, Spencer et al. 1993). Similarly, treatment of rats with the MR antagonist spironolactone resulted in an up-regulation of GR mRNA and protein in the hippocampus (Herman and Spencer 1998). These results suggest that glucocorticoids may be capable of regulating the extent of their own actions, not only through mediating negative feedback on the HPA axis, but also through regulation of their own receptors.

***Mechanisms of Corticosteroid Receptor-Mediated Gene Regulation:***

Endogenous glucocorticoids are released from the adrenal gland and circulate freely in the blood. Access of corticosteroids to receptors in the periphery is restricted by cortisol-binding globulin (CBG), which is thought to occupy as much as 95% of serum corticosteroids circulating over a 24-hour period (Dallman, Akana et al. 1994). However, CBG does not appear to be a determinant in corticosteroid access to hippocampal or cortical MR or GR, as CBG does not cross the blood-brain-barrier and expression of CBG in the brain of both rodents (Mopert 2004) and humans (Herbert, Pollak et al. 2006) appears to be restricted to discrete hypothalamic regions.

The lipophilic nature of corticosteroids allows them to freely diffuse across the plasma membrane and bind to cytoplasmic MR and GR. The ligand-activated receptors then translocate to the nucleus where they can either activate or repress transcription of corticosteroid-responsive genes via multiple mechanisms (Figure 1-4). The first level of transcriptional regulation involves binding of ligand-activated MR or GR to specific glucocorticoid response elements (GRE) in the promoter regions of corticosteroid responsive genes. MR or GR binding to GREs can result in either induction or repression of gene transcription, depending on the make-up of the specific GRE. GREs from positively regulated genes such as tyrosine-amino-transferase (TAT) and the long terminal repeat of the mouse mammary tumor virus (MMTV) have been found to contain consensus sequences similar to GGTACAnnnTGTTCT (Figure 1-4A). It is interesting to note here that while the DNA binding domain (DBD) of MR is nearly identical to GR, the ability of MR to synergistically activate transcription from multiple GREs does not equal that of GR. This is most likely due to limited homology in the N-terminal domains of MR and GR. Indeed, specific amino acid sequences termed synergy control motifs have been discovered in the N-terminal domains of multiple members of the steroid hormone superfamily and these motifs serve to dampen the more than additive transcriptional response of multiple GREs. While GR was found to contain 2 such motifs, MR was found to contain 4, potentially explaining this discrepancy (Iniguez-Lluhi and Pearce 2000). Repression of gene transcription through a negative GRE (nGRE) (Figure 1-4B) has also been reported, but a highly conserved consensus sequence has not been identified (Dostert and Heinzel 2004). An example of a gene that is repressed by GR/nGRE interactions is the POMC gene (Drouin, Trifiro et al. 1989).

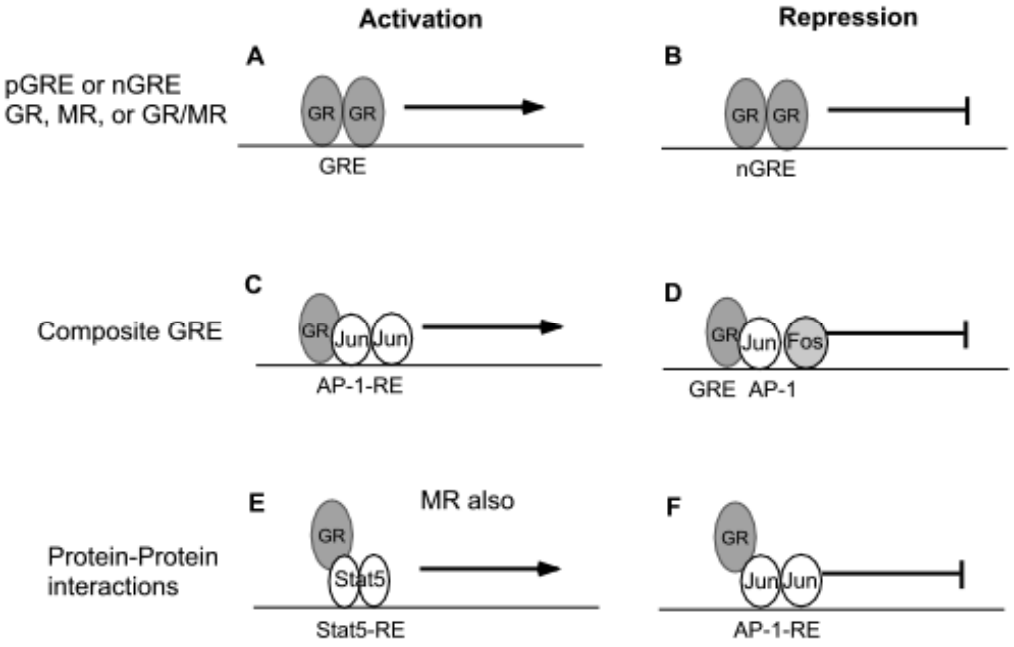


**Figure 1-4 Different mechanisms of transcriptional regulation of corticosteroid responsive genes by MR and GR.**

Additionally, it has been shown that the serotonin receptor 1a is repressed through binding of both MR and GR to an nGRE in its promoter (Ou, Storring et al. 2001).

The transcriptional response of a corticosteroid-responsive gene containing a positive or negative GRE can be futher modified by the ability of MR and GR to heterodimerize. In cells expressing only MR or GR, ligand-activated MR or GR translocates to the nucleus and binds GRE sites as a homodimer. In contexts where MR is co-expressed with GR, such as in the hippocampus, these two receptors have been shown to form heterodimers in addition to homodimers (Trapp, Rupprecht et al. 1994; Liu, Wang et al. 1995). Little is known about the rules that govern heterodimer vs. homodimer formation beyond stoichiometric considerations. It is known, however, that the transcriptional outcome of heterodimer formation is dependent on the cellular and/or promoter contexts. For example, in some instances, heterodimer formation appears to have synergistic effects on transcriptional activation with respect to either homodimer alone (Trapp, Rupprecht et al. 1994), whereas in other contexts the formation of heterodimers reduces the transcriptional activity of GR alone (Liu, Wang et al. 1995).

MR and GR also bind to GRE sequences in close proximity to or overlapping with sequences for other transcription factors, termed composite GREs (cGRE) (Figure 1-4C and D). When this occurs, the specific promoter sequence and the cellular context dictate the transcriptional outcome. The most prominent example comes from the cGRE of the proliferin gene, which has juxtaposed binding sites for GR and AP1 family members. In this example, GR can either activate or repress transcription dependent on the composition of the AP1 dimer, with cJUN/cJUN homodimers being activated and cFOS/cJUN heterodimers being repressed by GR (Diamond, Miner et al. 1990).

Interestingly, the corticosteroid receptor regulation of this cGRE is mediated exclusively by GR (Pearce and Yamamoto 1993). While no specific examples of MR involvement at a cGRE have been reported, this does not rule out the possibility that such regulation by MR can occur.

DNA binding-independent mechanisms of transcriptional regulation by MR and GR has also been proposed through protein-protein interactions (Figure 1-4 E and F). Most well known is the inhibition of the transcription factors NFkB and AP1 by GR (Jonat, Rahmsdorf et al. 1990; Almawi and Melemedjian 2002). Interactions between GR and the transcription factor Stat5 have been shown to enhance Stat5 dependent transcription (Stoecklin, Wissler et al. 1997), and subsequent data suggest the same may be true for interactions between MR and Stat5 (Stoecklin, Wissler et al. 1999).

The last level at which MR and GR are involved in the regulation of transcription involves the capacity of MR and GR to interact with other transcriptional coregulators. In general, ligand-bound steroid receptors interact with DNA and a large array of coregulators in a sequential and cyclical fashion that allows for the recruitment or stabilization of RNA polymerase II at the promoter and subsequent transcription initiation. More specifically, steroid receptor coactivators are thought to modify the chromatin around the hormone responsive elements by destabilizing the chromatin/histone interactions. This is hypothesized to "open" the DNA allowing for further recruitment of other transcription factors. Examples of steroid receptor coactivators that are capable of modifying chromatin through histone-acetyl-transferase (HAT) activity include CBP/p300, and members of a family of related proteins termed the p160 coactivators, represented by SRC-1, TIF-2, and pCIP family members. Other

coactivators are capable of destabilizing chromatin through histone-methyl-transferase activity (CARM1/PRTM1), or by destabilizing chromatin in an ATP dependent fashion (SWI/SNF) (Pascual-Le Tallec and Lombes 2005).

Conversely, MR and GR can also interact with corepressors that are capable of "closing" the DNA through their histone deacetylase (HDAC, eg. NCoR and SMRT) activity, or by interacting with proteins that are capable of directly (DAXX (Obradovic, Tirard et al. 2004)) or indirectly (PIAS1 (Pascual-Le Tallec, Simone et al. 2005)) inhibiting receptor activity. The interactions of coregulators with steroid receptors have been shown to occur in a ligand dependent fashion through binding to a region of the ligand binding domain (LBD) termed the activating function domain 2 (AF2) (Freedman 1999).

The MR also contains specific regions in its N-terminus that allow it to interact with coregulators differentially as compared to other steroid/thyroid hormone receptors. The N-terminus of MR is the longest in this superfamily and exhibits less than 15% homology when compared to other family members (Evans 1988). Two activation function domains have subsequently been found in the amino terminal region of MR, termed AF-1a and AF-1b (Fuse, Kitagawa et al. 2000).

Obradovic et. al. isolated three novel transcriptional regulators of MR and GR utilizing the AF-1 of MR: DAXX, FLASH, and FAF-1 (Obradovic, Tirard et al. 2004). While DAXX and FLASH influenced the transcriptional properties of MR and GR in a similar manner, FAF-1 was found to selectively stimulate MR mediated transcription while having no effect on GR. Perhaps the most interesting co-regulator discovered recently is ELL. This protein was shown to act as a selective coactivator of MR in that it

can potentiate MR mediated transcription and actually repress GR mediated transcription while having no affect on either PR or AR mediated transcription (Pascual-Le Tallec, Simone et al. 2005).

Hence, an analysis of the molecular mechanisms underlying MR- and GR-mediated transcriptional regulation suggests that these receptors may regulate corticosteroid-responsive genes in both similar and distinct manners. While a large body of work has analyzed the interactions of MR and GR homodimers with coregulators, the field is severely lacking in its analysis of MR/GR heterodimer interactions with coregulators. Overall, the data suggest that when MR and GR are co-expressed, the transcriptional regulation of corticosteroid responsive genes will be determined by the concentration of corticosteroid, the relative levels of MR and GR, and the expression profiles of other transcription factors and coregulators. All of these factors together may result in differential regulation of genes relative to the regulation derived from the activation of either MR or GR alone. In support of this notion, a recent study to identify glucocorticoid-responsive genes in the rat hippocampus using either low or high doses of corticosterone (to activate MR alone or MR and GR together) in ADX rats identified 203 genes that were regulated by either low or high doses of corticosterone but only 33 that were regulated by both doses of corticosterone (Datson, van der Perk et al. 2001). These data suggest that the activation of MR and GR together in the same cell, at varying ligand concentrations, may regulate distinct and yet partially overlapping sets of genes.

***Corticosteroid-Mediated Regulation of HPA axis Activity.***

As a classical physiological homeostatic system, the HPA axis is under negative feedback regulation by corticosteroids, the final product of the pathway. This negative feedback can occur at multiple levels as outlined in Figure 1-5. Corticosteroids have been proposed to directly repress the expression of POMC, the precursor of ACTH, in anterior pituitary corticotropes through multiple mechanisms, including a GR-mediated antagonism of Nur77 (Philips, Maira et al. 1997), and GR interactions with an nGRE in the POMC promoter (Drouin, Trifiro et al. 1989; Riegel, Lu et al. 1991). In the PVN of the hypothalamus, corticosteroids are known to down-regulate the expression of CRH and AVP. While these corticosteroid-mediated actions may include direct regulation of the AVP gene, the negative regulation of CRH expression by corticosteroids, however, is far more complex with both spatial and temporal domains (Watts 2005).

Corticosteroid-mediated negative feedback to the HPA axis is also provided by other brain regions outside of the hypothalamus. These are largely forebrain limbic regions that encompass the "anticipatory" branch of HPA axis activation and include the prefrontal cortex (PFC) and the hippocampus. As these regions do not project directly to the PVN, negative feedback is provided through multi-synaptic pathways that include relays through other brain regions (Herman, Figueiredo et al. 2003). Corticosteroid implants in the PFC reduce both the corticosterone and ACTH response to restraint stress in rats, however these implants have no effects on either basal AM or PM indices of HPA axis activity, suggesting that corticosteroid regulation is restricted to stress induced HPA activity (Diorio, Viau et al. 1993). Implants of corticosterone into the ventral hippocampus of the rat have been found to flatten the circadian rhythm of corticosteroid

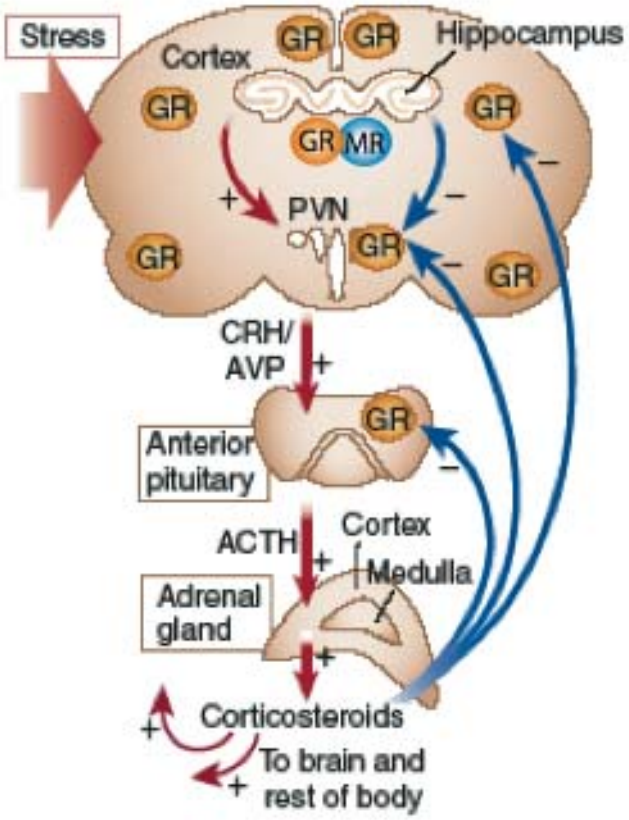


**Figure 1-5 Schematic of HPA axis including sites of negative feedback provided by GR and MR.**

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release (Slusher 1966), and implants of corticosterone into the dorsal hippocampus were found to decrease the adrenalectomy induced increase in ACTH (Kovacs and Makara 1988) suggesting that the hippocampus provides corticosteroid-mediated negative feedback to the HPA axis. In addition to negative feedback provided to the HPA axis by the PFC and the hippocampus, positive regulation is provided by the amygdala in a corticosteroid-responsive fashion. Shepard et al. found that implants of corticosterone to the amygdala resulted in an enhanced HPA axis response to a behavioral stressor, but had no effect on basal a.m. or p.m. corticosteroid levels (Shepard, Barron et al. 2003).

Interestingly, the glucocorticoid-mediated effects in all of these regions correspond with many of the electrical stimulation and lesioning studies discussed in the HPA Axis Activity section, highlighting the important role of glucocorticoid-mediated information transfer from these limbic regions to the PVN. All of these regions are known to express MR and GR, however, the hippocampus is notable in that it co-expresses the highest levels of both receptors. Because of the importance of negative feedback regulation and dysregulation in mood disorders, a broad array of techniques have been used to elucidate the individual roles of MR and GR in regulating basal and stress-induced HPA axis activity and anxiety-related behaviors. Below, I will review our current understanding of corticosteroid/MR/GR actions in these processes based on both pharmacological and genetic manipulation studies.

### *MR and GR Regulation of HPA Axis Activity: Pharmacological*

Under basal, resting conditions, corticosteroid levels in the plasma oscillate in a circadian fashion. At the trough of the circadian rhythm, corticosteroid levels are low and are thought to preferentially occupy MR, while at the peak, the hormone is elevated

to levels that occupy both MR and GR (Reul and de Kloet 1985). The differential occupancy of MR and GR is consistent with their differential binding characteristics with MR exhibiting a roughly 10-fold greater affinity for corticosteroids than GR. These data suggest that because of its high affinity and low capacity, activation of MR is essential for maintenance of the basal circadian rhythm, whereas activation of GR with increasing corticosterone levels is required for recovery of homeostasis via negative feedback (De Kloet and Reul 1987). Subsequent agonist and antagonist studies from several groups demonstrated the importance of MR activity in restraining circadian trough levels of corticosteroids (Ratka, Sutanto et al. 1989; Bradbury, Akana et al. 1991; Bradbury, Akana et al. 1994). However, it was also shown that MR activation was necessary, in conjunction with GR activation, in restraining peak HPA axis activity as well (Bradbury, Akana et al. 1994). In addition to studies in rats, it was also shown in humans treated with canrenoate, a selective MR antagonist, that MR activity affects basal HPA axis activity at both the circadian trough and peak (Dodt, Kern et al. 1993).

In response to stress, corticosteroid levels increase and participate in the classic negative feedback loop shown in Figure 1-5. Due to differences in testing paradigms and the types of antagonists used, the relative contributions of MR and GR in this negative feedback are not clearly defined. Two groups have shown that in response to mild stressors, such as exposure to a novel environment, blockade of MR in rats resulted in an increased or elongated stress response (Ratka, Sutanto et al. 1989; Pace and Spencer 2005). Weidenfeld et al. found in rats that administration of a GR antagonist resulted in an increased corticosterone response to photic stress, whereas blockade of MR did not, suggesting that only GR was necessary for restraining the HPA axis in response to photic

stress (Weidenfeld and Feldman 1993). In response to a more intense stressor, Spencer and colleagues showed that while blockade of either MR or GR alone had no effect on restraint stress-induced corticosterone levels at either the circadian trough or peak, if both receptors were blocked at the same time, stress induced corticosterone levels were significantly elevated above vehicle controls at both times of the day, suggesting that both receptors are important in negative feedback control in response to restraint stress (Spencer, Kim et al. 1998). In addition to acute stressors such as the ones mentioned, Spencer's group has also shown that blockade of MR, but not GR was sufficient to prevent the expression of habituation to repeated restraint in rats (Cole, Kalman et al. 2000). In humans, antagonist studies suggest a more prominent role for MR in restraining activated HPA activity as blockade of MR alone resulted in elevated cortisol levels in response to both exercise (Wellhoener, Born et al. 2004) and CRH challenge (Arvat, Maccagno et al. 2001). Overall, the literature suggests that MR and GR may both be important in restraining HPA axis activity to many, but not all types of stressors.

All of these studies utilized antagonists that were injected either in the periphery or in the brain (ie. intracereboventricular, icv) not permitting determination of region-specific effects of MR and GR blockade. In order to accomplish this, van Haarst and colleagues performed either icv or intra-hippocampal injections of MR and GR antagonists (van Haarst, Oitzl et al. 1997). It was found that while icv delivery of GR antagonists resulted in enhanced HPA axis activity, direct intra-hippocampal delivery actually resulted in a suppression of HPA axis activity. In contrast, both icv and intra-hippocampal delivery of MR antagonists resulted in enhanced HPA axis activity, suggesting distinct roles for hippocampal MR and GR in HPA axis regulation. The

amygdala and the PVN are regions that have also been shown to express both MR and GR and thus may be additional regions where the actions of MR and GR could provide either similar or differential control over HPA axis activity. Although corticosterone implants into the amygdala increase stress induced HPA axis activity and corticosterone applied locally to the PVN can inhibit adrenalectomy-induced increases in CRH and AVP production (Kovacs, Kiss et al. 1986), receptor-specific blocking studies have not been performed in these regions.

These studies suggest a rather complex regulation of the HPA axis by corticosteroids where the final integration at the level of the PVN can result in either enhanced or suppressed HPA axis activity. Importantly, these outcomes appear to be dependent on the levels of activation of both the specific brain region and the receptor that is activated.

### *Corticosteroid and MR/GR Regulation of Anxiety-Related Behaviors: Pharmacological*

It has been known for many years that activity of the HPA axis can modulate anxiety-related behaviors, but the actions of corticosterone on anxiety are quite complex. A recent review from Korte comprehensively organizes the vast literature in this field and finds that the effects of corticosterone on fear and anxiety are heavily dependent on whether the stressor is conditioned or unconditioned as well as on the phase of the corticosterone response (Korte 2001). The general effects of corticosterone have been studied in animal models of ADX and corticosterone replacement. ADX was shown to increase anxiety (Weiss, McEwen et al. 1970; Joffe, Mulick et al. 1972; File, Vellucci et al. 1979) and corticosterone replacement after adrenalectomy had anxiolytic effects (File,

Vellucci et al. 1979). Conversely, it has been reported that fear-induced immobility is reduced (decreased anxiety) in ADX animals and the immobility is restored upon corticosterone replacement (increased anxiety) (Bohus 1987), suggesting that the actions of corticosteroids on anxiety-related behaviors are dependent on context. Experimentally, a variety of different testing paradigms have been used to address this issue, including short and long-term exposure to corticosterone either systemically or in specific brain regions, as well as acute blockade of MR and GR with antagonists in either the whole brain (icv) or into specific brain regions. The effects of corticosteroids on measures of anxiety-like behavior not only display brain-region specificity, but there appear to be time dependent effects of corticosteroid exposure as well as differential effects of MR and GR activation.

When MR and GR are blocked acutely, the data suggest that they appear to be involved in modulating different aspects of fear and anxiety. For example, pharmacological MR inactivation specifically in the hippocampus suggests that it plays a permissive role in the expression of unconditioned fear, as blockade of hippocampal MR, but not GR, resulted in decreased anxiety-like behavior as measured by multiple tests including the elevated plus maze, the defensive burying test, and thigmotaxis in the open field (Bitran, Shiekh et al. 1998) as well as the light/dark box (Smythe, Murphy et al. 1997). In response to conditioned fear, activation of MR in the brain in general also appears to play a permissive role in the mediation of conditioned freezing behavior (Korte, de Boer et al. 1995) as well as the extinction of passive avoidance (Bohus and de Kloet 1981). Corticosteroids acting through GR appear to enhance acquisition, conditioning, and consolidation of an inescapable stressful experience (Korte 2001).

Activation of GR also appears to promote processes underlying fear potentiation, as blockade of GR in the brain (icv) prevents the fear potentiated decrease in time spent on the open arms of the Elevated Plus Maze (Korte, de Boer et al. 1995). These data coincide with a large number of lesioning studies in the rodent that suggest that the learning and memory of conditioned fear is derived from the amygdala but that the hippocampus contributes to unconditioned fear responses (McHugh, Deacon et al. 2004; Rosen and Donley 2006).

A number of experiments have been done to address chronic corticosterone treatments on anxiety-related behaviors, but differences in testing paradigms, including routes of administration, anxiety-tests utilized, and lengths of time of treatment, make it difficult to draw conclusions. For example, Ardayfio et al. found that chronic (17 days) but not acute (24 hr) corticosterone in the drinking water of mice *increased* anxiety-like behavior in mice in the light-dark emergence task (Ardayfio and Kim 2006). Using rats, Andreatini et al, found that subcutaneous injections of corticosterone dose dependently *decreased* anxiety-like behavior in the elevated plus maze within 2 hours of injection, but chronic injections of 14 days had no effect (Andreatini and Leite 1994; Andreatini and Leite 1994).

Different brain regions contribute to the expression of anxiety-related behaviors. Perhaps the best understood relationship between corticosteroids and anxiety occurs in the amygdala. A large body of research suggests that corticosteroids can modulate fear and anxiety-related behaviors through stimulation of CRH production in the amygdala (Korte 2001; Korte, Koolhaas et al. 2005). In sheep, stress induced levels of corticosteroids increase CRH levels in the amygdala in a manner that is dependent on GR

(Cook 2002). Corticosteroid implants into the amygdala of rats for 7 days increased anxiety-like behavior on the Elevated Plus Maze with a concomitant increase in amygdalar CRH expression (Shepard, Barron et al. 2000). In support of this, a recent study also found that corticosteroid implants into the amygdala of rats for 5 days increased anxiety-like behavior as measured by the Elevated Plus Maze. Interestingly, the corticosteroid effect on anxiety was blocked by co-implantation of both GR and MR antagonists (Myers and Greenwood-Van Meerveld 2007).

Overall, these data suggest that corticosterone can modulate anxiety-related behaviors. The manner in which this occurs, however, is complex. In the short-term, GR and MR activation may have different effects on aspects of consolidation of acquired information versus sensory integration underlying the evaluation of environmental information. Long-term elevations of glucocorticoids may have opposite effects on anxiety-related behaviors with respect to short-term elevations, but the mechanisms underlying this are not completely understood.

### *Genetic Manipulations of MR and GR: Effects on HPA axis and Anxiety*

In addition to these pharmacological methods, a large number of studies have been done that rely on genetic manipulation of MR and GR to determine their respective roles in HPA axis regulation and anxiety-related behaviors. These studies have been summarized in Table 1-3. For GR, these include transgenic mice with decreased GR expression (Pepin, Pothier et al. 1992), and transgenic mice with increased GR expression either globally (Reichardt, Umland et al. 2000), or in the forebrain specifically

**Table 1-3 HPA system dysregulation and behavioral symptoms in mice with targeted mutations of GR and MR**

| | Baseline HPA System | | | | Challenged HPA System | | | Behavior | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Hypothalamic CRH | Pituitary POMC/ACTH | Plasma ACTH | Plasma CORT | Stress ACTH | Stress CORT | dex/CRH test | Anxiety | Despair | Locomotion |
| Human depression | ⇑ | n.d. | ⇑ | ⇑ | ⇓ | ⇑ | ⇑ | ⇑ | ⇑ | |
| Human panic disorder | ⇔ | n.d. | ⇔ | ⇔ | ⇓ | ⇔ | (⇑) | ⇑ | ⇑ | |
| (1) Tg mice with ⇓ GR | ⇓ | ⇔ | ⇔ | ⇔ | ⇑ | ⇓ | ⇑ | ⇓ | ⇓ | ⇔ |
| (2) $GR^{null}$ mice | ⇑ | ⇑ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| (3) $GR^{NesCre}$ mice | ⇑ | ⇑ | ⇓ | ⇑ | ⇓ | ⇔ | n.d. | ⇓ | ⇓ | ⇔ |
| (4) $GR^{dim}$ mice | ⇔ | ⇑ | ⇔ | ⇑ | n.d. | n.d. | n.d. | ⇔ | ⇔ | ⇔ |
| (5) Tg - ⇑ GR (YGR) | ⇓ | ⇓ | ⇑ | ⇓ | ⇑ | ⇓ | n.d. | n.d. | n.d. | n.d. |
| (6) Tg - ⇑ GR (GRov) | ⇔ | ⇔ | ⇔ | ⇔ | ⇓ | ⇑ (prolonged) | n.d. | ⇑ | ⇑ | ⇔ |
| (7) $GR^{CaMKII/Cre}$ KO | ⇔ - AVP ⇑ | ⇔ | ⇑ | ⇑ | ⇑ | ⇑ | n.d. | ⇔ | ⇑ | ⇑ |
| (8) MR / mice | ⇑ | ⇑ | ⇓ | ⇑ | n.d. | n.d. | n.d. | ⇑ | n.d. | n.d. |
| (9) $MR^{CaMKII/Cre}$ KO | ⇔ | n.d. | n.d. | ⇔ | n.d. | ⇔ | n.d. | ⇔ | n.d. | ⇔ |
| (10) Tg - ⇑MR (MRov) | ⇔ | n.d. | n.d. | ⇔ | n.d. | ⇓ (females) | n.d. | ⇓ | n.d. | ⇔ |
| (11) Tg - ⇑MR (MRtg) | n.d. | n.d. | n.d. | ⇔ | n.d. | ⇔ | n.d. | ⇓ | n.d. | ⇔ |

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(Wei, Lu et al. 2004; Wei, Hebda-Bauer et al. 2007). Mouse models with a disrupted GR gene have also been generated either globally (Cole, Blendy et al. 1995), or in a forebrain-specific fashion (Tronche, Kellendonk et al. 1999; Boyle, Brewer et al. 2005). Transgenic mice have also been generated that contain a mutated DNA-binding domain (GRdim) (Reichardt, Kaestner et al. 1998). These studies demonstrate that GR plays an important role in modulating HPA axis activity and affect. Mice that are total GR deficient die shortly after birth demonstrating the importance of GR function in mouse development. It was found, however, that newborn mice homozygous for the $GR^{null}$ allele have enhanced transcription of both CRH in the hypothalamus and proopiomelanocortin (POMC) in the anterior lobe of the pituitary, confirming the role of GR-mediated negative feedback in the HPA axis (Gass, Kretz et al. 2000). Knock out or knock down of GR specifically in the brain results in elevated levels of corticosterone and decreased anxiety-related behaviors. Mice with a GR deficit specifically in the forebrain also show increased corticosterone levels (Boyle, Brewer et al. 2005). Behaviorally, these animals demonstrated increased depressive-related behaviors, such as anhedonia (defined as a decrease in the sensitivity of brain reward mechanisms), but showed increased locomotion without altered anxiety (Boyle, Kolber et al. 2006). When GR is overexpressed, many of the opposite phenotypes are seen relative to underexpression models. Mice with two extra copies of the GR gene show reduced expression of CRH and ACTH and show a fourfold reduction in the levels of circulating corticosterone; behavioral studies for anxiety have yet to be performed (Reichardt, Umland et al. 2000). When GR is overexpressed specifically in the forebrain, transgenic

mice show both increased anxiety and depression-related behaviors (Wei, Lu et al. 2004). These mice also exhibit a delay in the negative steroid feedback of the HPA axis and a mild cognitive deficit, similar to effects observed in aged mice (Wei, Hebda-Bauer et al. 2007).

Many fewer relevant mouse models exist for the study of MR in the brain. Mice were generated that lack MR globally (MR-KO), but these mice die within 10 days of birth due to renal sodium and water loss (Berger, Bleich et al. 1998). Although MR-KO mice can be rescued with exogenous salt injections, this requires daily handling of pups during the early postnatal time period, which can have profound effects on HPA axis activity and behavior (Anisman, Zaharia et al. 1998). With this caveat in mind, the salt-rescued mice were used to study the loss of MR on hippocampal morphology and neurogenesis (Gass, Kretz et al. 2000). It was found that MR-KO mice exhibit a decreased density of granule cells in the dentate gyrus of the hippocampus, indicative of neurodegeneration as well as decreased neurogenesis relative to both wildtype controls and mice that lack GR in the brain. These data suggest that MR may play an important role in hippocampal neurogenesis and cell survival, although it should be noted that these mice also have increased levels of corticosterone in the plasma which may confound the results. In other models, MR has been overexpressed (Ouvrard-Pascaud, Sainte-Marie et al. 2005) or underexpressed (Beggah, Escoubet et al. 2002) in the heart. Le Menuet et al. utilized the human MR promoter to drive expression of MR in the mouse, but only studied MR function relative to the kidney and the heart (Le Menuet, Isnard et al. 2001).

More recently, mice that lack MR in the forebrain were generated using the Cre-LoxP recombination system with the CaMKIIα promoter being used to drive expression

of Cre recombinase (Berger, Wolfer et al. 2006). These mice show no changes in HPA axis activity either basally or in response to stress. They also do not exhibit any changes in anxiety-related behavior, although they do exhibit a learning deficit in the Morris Water Maze (MWM) and the radial maze. While these mice have a complete loss of MR in the forebrain, they also exhibit a compensatory increase in GR expression in the hippocampus that may confound interpretations. Interestingly, a recent study using these animals found that MR is required for stress levels of corticosterone to rapidly modulate glutamate transmission in region CA1 of the hippocampus (Karst, Berger et al. 2005). This suggests that the classic cytoplasmic MR may be capable of mediating fast non-genomic cellular responses in addition to its role in the traditional genomic pathway, perhaps by associating with plasma membrane bound proteins capable of transducing traditional intracellular signaling cascades.

Concurrent with the published data presented in chapter 2 of this thesis, a similar mouse model was published that also overexpresses MR in the forebrain (Lai, Horsburgh et al. 2007). Similar to the transgenic animals generated for this thesis, these transgenic animals exhibit a decrease in anxiety-like behavior. No changes were seen in HPA axis activity either basally or in response to stress, although only male animals were used for analysis. These mice also exhibited a mild enhancement in spatial memory retention relative to wildtype controls. Interestingly, these transgenic mice also showed a decrease in cell death in region CA1 of the hippocampus in response to ischemic insult, suggesting that MR can play a neuroprotective role. Thus altered levels of brain MR have been suggested to affect neurogenesis, learning, and neuroprotection while its roles in HPA function and anxiety have not been clearly established.

### *Corticosteroids and Hippocampal Structure and Function*

As mentioned previously, disturbances in HPA axis activity underlie multiple mood disorders including major depressive disorder and multiple anxiety disorders, often leading to excessive levels of plasma corticosteroids. The excessive levels of corticosteroids are thought to contribute to the decreased structural and functional integrity of corticosteroid-responsive brain regions, particularly the hippocampus, but also including regions of the cortex. Recent data have shown that the subtypes of depression with the highest rates of hypercortisolism are most often associated with atrophy of the hippocampus (Sheline, Sanghavi et al. 1999). As the hippocampus is essential for learning and memory, it is not surprising that both major depression and anxiety disorders often include defects in declarative learning and memory (Austin, Mitchell et al. 2001; Airaksinen, Larsson et al. 2005). Corticosteroids are known to have potent effects on many aspects of the structure and function of the hippocampus, and thus serve as a primary link between hippocampal structure and function and affective disorders.

Repeated stress or repeated exposure to elevated levels of corticosteroids over several weeks lead to a retraction of dendrites in region CA3 of the hippocampus (Woolley, Gould et al. 1990; Watanabe, Gould et al. 1992; Sousa, Lukoyanov et al. 2000; Conrad CD 2007). Four weeks of chronic unpredictable stress (CUS) resulted in impairments in learning and memory in addition to its effects on CA3 dentritic morphology (Sousa, Lukoyanov et al. 2000). The chronic corticosterone treatment groups had milder alterations of CA3 dendritic processes than the 4 weeks of CUS, and also exhibited milder learning deficits, suggesting that factors other than the

corticosterone produced during stress may contribute to morphological and behavioral changes.

New neurons are continually being born in the mammalian dentate gyrus, and this process of neurogenesis has also been shown to be highly regulated by corticosterone. Rats treated acutely with corticosterone showed a significant decrease in the number of newborn neurons, while ADX results in a large increase in neurogenesis (Cameron and Gould 1994). Additionally, physiological alterations in corticosterone levels, such as acute and chronic stress are known to suppress hippocampal neurogenesis (Gould and Tanapat 1999). The activation of GR is clearly implicated in the suppression of neurogenesis in the dentate gyrus. High levels of corticosterone given to either adrenalectomized (Wong and Herbert 2005) or adrenal-intact rats (Mayer, Klumpers et al. 2006) resulted in a suppression of proliferation that was normalized by antagonism of GR. Other evidence suggests that the GR agonist dexamethasone can inhibit neurogenesis both *in vivo* and *in vitro* (Kim, Ju et al. 2004). The effects of MR activation on neurogenesis are not as clear. Several groups have shown that MR activation can suppress the increased proliferation that accompanies adrenalectomy (Wong and Herbert 2005; Krugers, van der Linden et al. 2007) in a manner similar to GR. In contrast, however, it has also been shown that aldosterone can further enhance the increased proliferation that accompanies adrenalectomy (Fischer, von Rosenstiel et al. 2002). In support of this positive effect of MR activation on neurogenesis, genetic disruption of MR leads to impaired neurogenesis in the hippocampus, whereas brain specific disruption of GR had no effects on hippocampal neurogenesis (Gass, Kretz et al. 2000). In a series of *in vitro* experiments, primary hippocampal cultures treated with aldosterone showed

enhanced neurogenesis that could be blocked with the MR antagonist spironolactone. In contrast, cultures treated with dexamethasone showed decreased neurogenesis, an effect that could be blocked by mifepristone (Fujioka, Fujioka et al. 2006).

Finally, long-term elevations in corticosteroid levels can have negative effects on neuroplasticity and cell survival, a phenomenon that has been referred to as glucocorticoid endangerment (Sapolsky 1985). Region CA1 of the hippocampus is particularly sensitive to glutamate toxicity induced by neurological insult and excess corticosteroids make this region more vulnerable to damage (Sapolsky 1985; Sapolsky and Pulsinelli 1985). While there is some evidence that corticosteroids can be directly toxic to neurons, a number of other glucocorticoid actions have been identified that may contribute to the ability of corticosteroids to exacerbate neuronal death. These include increasing the basal free intracellular cytosolic calcium concentrations (Elliott and Sapolsky 1992; Elliott and Sapolsky 1993) and inhibiting glucose transport (Tombaugh and Sapolsky 1992; Lawrence and Sapolsky 1994), outlining a general scheme where the glucocorticoid-mediated disturbance of energy homeostasis in the cell makes it more vulnerable to toxic insults such as excess glutamate.

As the hippocampus is richly endowed with both GR and MR, it is of major importance to understand the relative contributions of each receptor to this process of glucocorticoid endangerment. There is substantial evidence that the glucocorticoid endangerment effect is mediated by GR (Herman and Seroogy 2006). Interestingly, while extensive GR activation appears responsible for many of the deleterious consequences of glucocorticoids, MR activation appears to provide a neuroprotective role. Overexpression of MR in the hippocampus provides protection from ischemic

insult relative to wildtype controls (Lai, Horsburgh et al. 2007). Overexpression of MR in the neuronal cell line PC12 also provides protection from various neurological insults (Lai, Seckl et al. 2005). Crochemore and coworkers found that MR activation with aldosterone can prevent the dexamethasone induced apoptosis of primary hippocampal cell cultures (Crochemore, Lu et al. 2005). Similarly, McCullers and collegues, found that rats treated with an MR antagonist showed substantial exacerbation of kainite-induced neurotoxicity in subfield CA3 of the hippocampus, whereas in contrast, GR blockade did not affect cell loss (McCullers and Herman 2001). Additionally, the increased neurodegeneration after MR antagonism was associated with a decrease in expression of Bcl-2, an anti-apoptotic protein, in this same subregion (McCullers and Herman 1998). Hence, numerous studies suggest that MR may provide neuroprotective effects in response to insult through modulation of anti-apoptotic mechanisms.

The activity of MR also appears to play a supportive role in cell survival. Removal of corticosteroids by adrenalectomy results in a large amount of cell death in the dentate gyrus. Very low levels of corticosteroids or administration of an MR agonist is sufficient to prevent this neurodegeneration, suggesting that there are trophic actions of corticosteroids that are mediated by MR (Woolley, Gould et al. 1991; Sloviter, Sollas et al. 1993).

On a cellular level, glucocorticoids are intimately involved in modulating multiple properties relevant to learning and memory and information transfer out of the hippocampus. Levels of glucocorticoids equivalent to a stressor decrease long-term potentiation (LTP) whereas low levels of corticosteroids enhance LTP (McEwen and Sapolsky 1995). Similarly, low levels of corticosterone can enhance excitability of

pyramidal neurons in the hippocampus and higher levels of hormone result in decreased neuronal excitability (Joels and de Kloet 1991; Joels and de Kloet 1992). Interestingly, under conditions of ADX, when no corticosteroids are present, a similar cellular phenotype is observed as under high levels of corticosteroids. These inverted U shaped patterns can be explained by the relative occupations of the two corticosteroid receptors present in the hippocampus. Studies using specific agonists and antagonists for MR suggest that the activity of this receptor is responsible for increased LTP and increased neuronal excitability. Conversely, higher levels of corticosteroids, activating both MR and GR, and specific agonists for GR decrease LTP and neuronal excitability (Joels and de Kloet 1991; De Kloet, Vreugdenhil et al. 1998).

As one would predict from the LTP data, lower levels of glucocorticoids commensurate with low to moderate stress have been shown to enhance cognition in several learning and memory paradigms, whereas more severe stressors and higher levels of glucocorticoids disrupt it (Luine, Villegas et al. 1994; Bodnoff, Humphreys et al. 1995; McLay, Freeman et al. 1998). It should be stressed here, however, that these effects of corticosteroids on learning and memory are time dependent, as acute glucocorticoid treatment in close relation to training has been shown to potentiate memory in a dose-dependent fashion (De Kloet, Vreugdenhil et al. 1998). These findings highlight that corticosteroids modulate information processing in a time- and context-dependent fashion.

Many studies have been performed to determine the individual roles of MR and GR in learning and memory. While these studies clearly implicate MR and GR in cognitive function, an overall conclusion is difficult to ascertain as a wide range of

testing paradigms and conditions have been used. Acute blockade of MR and GR during different phases of morris water maze (MWM) training has implicated MR activity in search escape strategies, whereas GR activity was implicated in the consolidation of spatial memories (Oitzl and de Kloet 1992). However, here too, there seems to be a time dependent effect of receptor blockade. For example, chronic blockade of MR for 12 days impaired spatial learning in the MWM (Yau, Noble et al. 1999). Similarly, repeated blockade of MR, but not GR resulted in impaired food-rewarded spatial learning in the hole-board task (Douma, Korte et al. 1998).

Overall, glucocorticoids have been shown to heavily influence the structure and function of the hippocampus. Research suggests that the actions of MR and GR in these processes may be separable, mediating differential effects on cellular properties such as LTP and ion conductances, as well as differential effects on cell survival and learning and memory.

***Thesis Summary:***

This introduction has described how corticosteroids, acting through MR and GR, are critical to the maintenance of homeostasis in mammals. The similarities and differences in MR and GR properties, including differential ligand sensitivities, expression patterns, and modes of transcriptional activation/repression suggest they have both independent and overlapping roles in regulating allostatic mechanisms that comprise the "stress response". The HPA axis is a highly dynamic system and corticosteroid actions are not strictly coincidental with the level of hormone; levels of the individual receptors also contribute to the final biological output. MR and GR can function as

monomers, homodimers, or heterodimers when co-expressed, with very different outcomes based on the concentration of hormone and the level of each receptor. Thus, the balance in MR and GR levels and activation is hypothesized to be a significant factor in proper cellular, physiological and behavioral responses to corticosteroids. Several endogenous systems of altered MR activity suggest that this is indeed the case. For example, the Lewis rat exhibits higher levels of hippocampal MR relative to the Wistar rat from which it is derived and shows lower evening basal and stress-induced HPA axis activity (Oitzl, van Haarst et al. 1995). Different strains of aged rats have decreased MR in the hippocampus relative to GR, and these rats show increased basal and/or stress induced HPA axis activity (Morano, Vazquez et al. 1994; Van Eekelen, Oitzl et al. 1995). Interestingly, in both rats that have received chronic unpredictable stress and in suicide victims with a history of depression, the expression levels of MR has been shown to be decreased, leading overall to an imbalance in the MR/GR ratio (Lopez, Chalmers et al. 1998). These data support the hypothesis that a balance in the activation of MR and GR in forebrain regions such as the hippocampus is an important process in the maintenance of proper homeostasis.

Previous studies attempting to elucidate the role of MR in anxiety-related behaviors and in the regulation of HPA axis have focused on acute blockade of MR with receptor specific antagonists. While this method has provided invaluable information regarding short-term signaling consequences, it does not provide information regarding long-term alterations in MR signaling. Thus, chapter II of this thesis addresses long-term alterations in MR signaling through the generation of transgenic mice that specifically overexpress MR in the forebrain. **We hypothesized that overexpression of MR in the**

**forebrain would decrease basal and stress induced HPA axis activity. We also hypothesized that overexpression of MR would alter anxiety-related behaviors.** These studies were published in the manuscript "Mineralocorticoid receptor overexpression in forebrain decreases anxiety-like behavior and alters the stress response in mice" PNAS (2007) 104(11):4688-4693.

Corticosteroids are also known to influence learning and memory and neurogenesis in the hippocampus but the role of MR in this process is currently unclear. As such, we were interested in determining the effects of overexpression of MR in the hippocampus on learning and memory and neurogenesis. **We hypothesized that overexpression of MR in the hippocampus would enhance hippocampal-dependent learning and memory and increase proliferation of dentate gyrus granule cells.** These studies are presented as appendices following chapter 2.

High levels of corticosteroids can have deleterious effects on the structure and function of the hippocampus. The mouse hippocampal cell line HT-22 has been used as a model system to study glucocorticoid endangerment of neurons. This cell line expresses GR but not MR, and glucocorticoid pre-exposure of these cells has been shown to exacerbate glutamate toxicity. Recent studies suggest that MR activation may play a neuroprotective role, promoting cell survival in response to various insults. To address this neuroprotective role, we generated stable clones of HT-22 cells that express varying levels of MR in addition to the GR already present in the cell. **We hypothesized that the expression of MR in HT-22 cells could attenuate the ability of corticosterone to exacerbate glutamate toxicity.** Data suggesting a potential neuroprotective role for MR is presented in chapter 3.

MR and GR function in large part as ligand-activated transcription factors that control the expression of gene networks. The relative levels of these receptors within the same cell have been shown to influence transcriptional regulation of corticosteroid-responsive genes in manners that are distinct from when either MR or GR is present alone. Thus, HT-22 cells and a stable clone of HT-22 cells that expresses MR in addition to GR have been used for microarray analysis to determine the effects of MR and GR activation together on global corticosteroid-responsive gene transcription relative to when GR alone is activated. **We hypothesized that the activation of MR and GR together in the same cell would significantly alter the expression patterns of both similar and different corticosteroid-responsive genes relative to GR activation alone.** Data summarizing these findings are presented in chapter 4.

Overall, disturbances in the structure and function of the hippocampus are hypothesized to underlie some depressive and anxiety disorders. Glucocorticoids have been shown to heavily influence cellular properties that may affect the structure of the hipppocampus, as well as physiological and behavioral processes that are dependent on the hippocampus. As much less is known about the function of MR in mediating the effects of glucocorticoids in the central nervous system, the goal in this thesis has been to address the role of MR in these processes using both *in vivo* techniques to understand physiological and behavioral consequences of MR action, and *in vitro* techniques to understand cellular consequences of MR action.

## Chapter 2

## Mineralocorticoid Receptor Overexpression in Forebrain Decreases Anxiety-Like Behavior and Alters the Stress Response in Mice

### *Introduction:*

The hypothalamic-pituitary-adrenal (HPA) axis controls the production and release of adrenal glucocorticoids (GC) across the daily circadian rhythm and in response to stress. The major functions of GC within the HPA axis are twofold: 1) alter metabolic processes in a manner that provides the energy needed to combat the stressful stimuli; 2) recover homeostasis. These functions are essential to the well-being of the organism and dysregulation of the axis can result in potentially deleterious consequences. Indeed, evidence suggests that multiple mood disorders, including anxiety conditions and major depression, are associated with chronic alterations in the circulating levels and circadian rhythm of GC (Chrousos and Gold 1992; De Kloet, Vreugdenhil et al. 1998; Akil 2005). Furthermore, clinically efficacious antidepressant treatment is often accompanied by normalization of the HPA axis, underscoring the importance of HPA axis function in control of affect (Lopez, Chalmers et al. 1998).

The actions of GC are mediated by two corticosteroid receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). MR and GR function as ligand-activated transcription factors that reside in the cytoplasm, dimerize

upon ligand binding, translocate to the nucleus and exert transcriptional control, either positive or negative, over glucocorticoid responsive genes. MR and GR have different affinities for corticosterone (Kd = 0.5nM for MR and 5nM for GR), and thus are differentially activated throughout the circadian rhythm and during times of stress. GR is widely expressed throughout the brain, whereas MR expression, although present in various brain regions, is found predominantly in the hippocampus in rodents. Interestingly, MR is highly expressed in cerebral cortex of squirrel monkeys and humans, suggesting additional roles for cortical MR in primates (Patel, Lopez et al. 2000; Watzka, Bidlingmaier et al. 2000). In brain regions where MR and GR are co-expressed, they can heterodimerize, further increasing the complexity by which the receptors can signal (Trapp and Holsboer 1996). While numerous studies have shown that MR and GR act synergistically in HPA axis inhibition, other work has suggested that the actions of MR and GR can be antagonistic. For example, MR and GR mediate opposite effects on ion conductances in hippocampal pyramidal neurons (Joels and de Kloet 1990) and can interact with co-activators in distinct manners (Meijer 2002). These differential mechanisms of action allow for a diverse response to corticosterone even within the same cell. Because of the importance of corticosteroid-mediated activity in anxiety and depressive disorders, it is critical to understand the individual roles of forebrain MR and GR in HPA axis regulation and control of affect.

The traditional view posits that, because of its high affinity and low capacity, activation of MR is essential for maintenance of the basal circadian rhythm, whereas activation of GR is required for the stress response and the subsequent recovery of homeostasis via negative feedback (De Kloet and Reul 1987). However, more recent

studies suggest that MR activity is required in conjunction with GR at the peak of the circadian rhythm and during times of stress (Bradbury, Akana et al. 1994) or that MR alone can mediate negative feedback in response to various challenges (Reul, Probst et al. 1997; Arvat, Maccagno et al. 2001; Wellhoener, Born et al. 2004). These results are consistent with MR antagonist studies in humans that demonstrate a clear role for MR in HPA regulation and increased MR function in depression (Young, Lopez et al. 1998; Young, Lopez et al. 2003). In contrast, recent findings in forebrain specific MR-deficient mice show no alterations in HPA axis activity under basal or stress activated conditions (Berger, Wolfer et al. 2006). Clearly, additional studies are required to elucidate the role of MR both in HPA function and in the modulation of affective responses.

In addition to the roles of MR and GR in HPA axis regulation, research over the last decade has suggested a potential role of these molecules in modulation of anxiety. The evidence is clearest for GR, as genetic manipulation has shown that decreased GR signaling in the brain results in decreased anxiety-like behavior (Montkowski, Barden et al. 1995; Tronche, Kellendonk et al. 1999), whereas overexpression of GR in the forebrain results in increased anxiety-like behavior (Wei, Lu et al. 2004). MR antagonist application directly into the hippocampus (Smythe, Murphy et al. 1997; Bitran, Shiekh et al. 1998) or brain in general (Korte, de Boer et al. 1995) suggest a similar anxiogenic role for MR either exclusively or in conjunction with GR. However, these effects are transient and do not address whether sustained alterations of MR would be anxiogenic or anxiolytic.

To specifically examine the role of MR in corticosteroid-mediated basal and stress induced feedback regulation of the HPA axis and/or anxiety-like behaviors, we

generated transgenic mice that overexpress forebrain MR (MRov). In this system, the calcium-dependent calmodulin kinase IIα (CaMKIIα) promoter is used to direct expression of MR specifically to forebrain regions. This promoter is not expressed prenatally, preventing overexpression of MR during development. In contrast to the forebrain specific MR-deficient mice, we find that overexpression of MR in the forebrain does impact stress-induced regulation of the HPA axis in female mice, and results in a reduction in anxiety-like behavior in both male and female mice. These data highlight the differential roles for MR and GR in the modulation of anxiety-related behaviors and suggest a more prominent role for MR in HPA axis regulation.

***Materials and Methods:***

**Complementary DNA (cDNA) Cloning.** To isolate a mouse brain MR cDNA, a 700 bp StuI/XbaI fragment from the rat MR cDNA (provided by Paresh Patel, University of MI) was used to screen a UniZAP XR mouse brain cDNA library (Stratagene, La Jolla, CA). Approximately 850,000 phage were screened under conditions previously described (Cortright, Nicoletti et al. 1995). In vivo excision of the pBluescript phagemids containing the hybridization-positive cDNA clones was performed according to the manufacturer's protocol. Eight clones were isolated and characterized by restriction digests and DNA sequence analysis. The University of Wisconsin Genetics Computer Group (Madison, WI) Sequence Analysis Software Package was utilized for sequence alignments. The resulting full -length cDNA clone pMR-2A was used for subsequent experiments.

**Generation of 3XFlag-Tagged MR and Transgenic Mice.** The endogenous MR translation start codon was replaced with a Kozak sequence and an N-terminal 3XFlag-tag sequence (ACC**ATG**GACTACAAAGACCATGACGGTGATTATAAAGAT CATGACATCGATTACAAGGATCATGATGGT) using a PCR based cloning strategy. A BsgI/PshAI fragment containing the 3XFlag-tag N-terminus was used to replace the unmodified BsgI/PshAI site of pMR-2A to generate pMR2AFlag. The MR2AFlag coding sequence was cloned into the EcoRV site of pNN265, which flanks the insert with a 5'UTR+intron and a 3' UTR+polyadenylation signal sequence, to create pNN265-MR2AFlag. The full-length non flag-tagged MR cDNA was also cloned into pNN265, which allows for the expression of the inserts from a minimal CMV promoter. The NotI fragment of pNN265-MR2AFlag containing the MR2AFlag coding sequence plus surrounding 5' and 3' regulatory sequences was cloned into the NotI site of pMM403, which places the fragment under the control of the CaMKIIα promoter (Mayford, Bach et al. 1996), to generate pMM403-MR2AFlag. pMM403-MR2A-Flag was digested with Sfi I and the 14.5kb insert was purified and injected into (C57Bl/6J x SJL) F2 mouse eggs by the University of Michigan Transgenic Animal Core. Ten transgenic founders were bred to C57BL6J mice, and 7 lines were established. Transgenic mice were maintained as hemizygotes and wildtype littermates were used as controls in all experiments. Genomic DNA prepared from tail biopsies was screened for the presence of the transgene by PCR. The transgene primers for genotyping are: 5'-GTT CTT CTC TTG GAC CTG CAG-3' and 5'-CCG CCA GTC CAG CGG TAA G-3'. The β–globin primers are 5'-CCA ATC TGC TCA CAC AGG ATA GAG AGG GCA GG-3' and 5'-CCT TGA GGC TGT CCA AGT GAT TCA GGC CAT CG-3'. PCR reactions contained both sets of primers (30

cycles of 1min @ 94, 1.5 min @65, and 1.5 min @72 C, followed by a terminal 10 min extension @72 C). PCR products are 727 bp for the MR transgene and 450 bp for β-globin internal control.

**Animal Husbandry:** Mice were maintained on a 14:10 light/dark schedule with lights on between 6:00 and 20:00. Mice were given food (5008 mouse chow, 9% fat content; Purina Mills, Inc., St. Louis, MO) and water ad libitum. C57BL/6J (The Jackson Laboratory, Bar Harbor, ME) mice were bred at the University of Michigan. All procedures using mice were approved by the University of Michigan Committee on Use and Care of Animals. All experiments were conducted in accordance with the principles and procedures outlined in the National Institutes of Health guidelines for the Care and Use of Animals.

**Cell Culture and Transfection.** CV-1 and Cos-1 cells were maintained in DMEM Invitrogen) containing 10% heat-inactivated FCS (Hyclone, Logan, UT) at 37C and 5% $CO_2$. To examine the possible effects of the addition of the 3X Flag tag to the N-terminus of MR, both transactivation and binding assays were performed. Twenty-four hours prior to experiments, $2.5x10^5$ or $3\text{-}5x10^5$ cells were plated in media containing 10% charcoal-stripped serum on 6 cm plates (for transactivation assays) or 10 cm plates (for binding assays), respectively. For the transactivation assays, CV-1 cells were transfected with 5μg of 5GRE-Luc reporter plasmid, 1μg CMV β-galactosidase, and 1μg of either pNN265-MR2aFlag or pNN265-MR2a using Fugene-6 Transfection Reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol. The

CMVβgal expression vector was included for normalization purposes. Twenty-four hours post transfection cells were either left untreated or treated with either 10nM aldosterone or 100nM corticosterone (Sigma-Aldrich, St. Louis, MO. Cells were harvested 24 h later in cold 1X PBS, pelleted, and lysed in 100µl lysis buffer [0.25 M Tris (pH 8.0), 0.1 mM EDTA, 15 mM MgSO4, 1 mM dithiothreitol (DTT), and 1% Triton X-100] and incubated on ice 10 min. Lysates were centrifuged at 10,000 rpm for 10 min at 4 C, and 10µl of each supernatant was added to 100µl of luciferase assay buffer (20 mM Tris (pH 8.0), 1.07 mM magnesium carbonate, 2.67 mM magnesium sulfate, 0.1 mM EDTA, 33.3 mM DTT, 0.27 mM Coenzyme A (Calbiochem, San Diego, CA), 0.53 mM ATP, and 0.47 mM luciferin (Analytical Luminescence, Ann Arbor, MI)) and assayed for 10 sec in a Turner 20/20 (Promega, Madison, WI) luminometer. β–gal activity was determined as previously described (Seasholtz, Thompson et al. 1988) For the binding assays, Cos-1 cells were transfected with 5µg of either the pMM403-MR2AFlag or pMM403-MR2A expression constructs. 40 hours later, cells were harvested and resuspended in TEMDG (20 mM Tris/1 mM EDTA/20 mM sodium molybdate/5 mM DTT/10% glycerol/10% Sigma Protease Inhibitor Cocktail, pH 7.6 at 4°C) binding buffer. After a brief sonication (setting 4 for 5 seconds with a Branson Sonifier 150), samples were centrifuged at 4°C at 16,100Xg for 30 min. Lysates were incubated, in triplicate, for 20 hours at 4°C with 7 serial 1:2 dilutions of $^{3}$H corticosterone plus or minus an excess of unlabeled corticosterone for determination of nonspecific binding. Bound and free [$^{3}$H]corticosterone levels were separated by addition of an ice-cold dextran/charcoal suspension in TEMDG (0.05/0.5%). The tubes were incubated 10 min at 0-2°C and centrifuged. The supernatants were removed and counted in a Packard

scintillation counter at 45% efficiency. Protein content was measured by method of Bradford (Bradford 1976) using BSA as a standard, and the receptor densities were expressed as fmol/mg protein. The dissociation constant ($K_d$) of MR was calculated by Scatchard analysis.

**Immunoprecipitations and Western Blots.** To analyze whole-cell MR2A-Flag protein levels, tissues were homogenized in TEMS buffer (50mM Tris, 1mM EDTA, 6mM $MgCl_2$, 10% sucrose, pH 7.2 @ 4C) in a glass dounce homogenizer on ice and then sonicated briefly (5 sec setting 4 of Branson sonifier 150). Lysates were centrifuged at 4°C at 16,100Xg for 30 min to remove cellular debris; supernatants were assayed for protein concentration. One hundred μg of protein/sample (exceptions: pituitary, 40μg, and hypothalamus, 80μg) was immunoprecipitated with 1 μg of a polyclonal anti-flag antibody (Sigma) in a total volume of 200μl for 2 hours at 4°C on a rocking platform. Twenty μl of re-suspended protein G coated beads (Santa Cruz, Santa Cruz, CA) was then added to each sample and incubated overnight as above. Samples were centrifuged at 1000Xg at 4°C. The tissue lysate was removed and saved for loading control immunoblot analysis, and the protein G coated beads were washed with cold PBS (repeated 2x). Samples were then resuspended in PBS and brought to a final volume of 20μl with 5x SDS loading dye, boiled 5 minutes, and iced 5 minutes. Resuspended immunoprecipitates were separated on a 10% SDS-PAGE gel and transferred onto PVDF membranes with a Bio-Rad Trans-Blot SD semi-dry transfer system. For immunoblots, membranes were blocked in a buffer containing 20mM Tris pH 7.5, 150mM NaCl, and 0.1% Tween 20 (TBST) + 5% non-fat dry milk (NFDM), for 1hr at room temperature,

then incubated for 1hr at room temperature with an HRP-linked anti-Flag antibody (Sigma) at 1:6000. For control immunoblot analysis, 10 μl of the tissue lysates were used for Western blotting with a monoclonal anti-β-tubulin antibody (Sigma) and an HRP linked donkey-anti mouse secondary antibody as described above. Immunoblots were visualized with ECL Plus (Amersham Biosciences, GE Healthcare) according to the manufacturer's protocol. Control immunoblot analysis revealed comparable levels of β-tubulin signal in similar brain regions from the different transgenic lines and wildtype control (data not shown).

***In Situ* Hybridization.** *In situ* hybridization was performed as previously described (Herman, Wiegand et al. 1990). The high-stringency wash for all probes was done in 0.1x SSC at 65°C. Riboprobes for MR were synthesized from pGem11zASMR, which contains a 277bp StuI/NcoI fragment of the mouse MR N-terminal region containing nucleotides 940-1217 from accession number XM_983321. pGem11zASMR was linearized with EcoRI and antisense riboprobes synthesized with SP6. Riboprobes for CRH were synthesized from pGem4zPST578 (Seasholtz 1991) that contains a 578 bp PstI fragment of the mouse CRH exon II. PGem4zPST578 was linearized with HindIII and antisense riboprobes synthesized with SP6 RNA polymerase. Antisense riboprobes for hnCRH were synthesized from pGem4zKpn/PvuII442, containing CRH intronic sequences (nucleotides 569 to 1011 from accession number AY128673), using SP6 RNA polymerase and plasmid that was linearized with HindIII. Riboprobes for mouse GR were synthesized from mGR600, which contains a 596 bp Bst217I/XbaI mouse cDNA fragment containing nucleotides 1-596 from NM_008173. EcoRI was used to linearize

mGR600 and antisense probes were synthesized with T3 RNA polymerase. r5HTr1a riboprobes were synthesized from a 905 bp MscI/PvuII rat cDNA fragment containing nucleotides 212-1117 from NM_012585. The plasmid was linearized with EcoRI and antisense probes were generated with SP6 RNA polymerase. The probe used to specifically detect expression of the transgene (tg probe) was generated by PCR using primers 5'-GGT GTC CAC TCC CAG GTC CAA CTG C-3' and 5'-CAT TCA GAA AAC TGA ATT AAA TCT A-3' and pMM403-MR2A-Flag as DNA template. This 190bp PCR product encompasses the vector-encoded 5' UTR region that will be specific to the transgene mRNA only and will not hybridize to any region of MR. This plasmid was linearized with EcoRV and SP6 RNA polymerease was used to generate antisense probes. All riboprobes were double labeled with [$^{35}$S]UTP and [$^{35}$S]CTP (Perkin Elmer) using standard transcription reaction conditions. Radiolabeled cRNA robes were purified from free nucleotides using Bio-Rad Micro Bio-Spin 6 columns. Semi-quantitative analysis of autoradiogram images was performed using the MCID image analyses system and Image J (NIH). Optical density measures were corrected for background to yield mean grey levels which were multiplied by the area sampled to produce an integrated density measurement.

**In Vitro Receptor Autoradiography.** [$^{3}$H]-8-Hydroxy-2-(N,N-di-N-propylamino-tetralin) ([3H]-8-OH-DPAT) binding to detect 5HT-1a receptors was performed as previously described (Lopez, Chalmers et al. 1998). Sections were apposed to tritium sensitive film (Amersham) and exposed at room temperature for 13 days. Semi-quantitative analysis of autoradiogram images was performed using Image J.

**Behavioral Testing.** All behavioral tests were performed with an n = 14-20 animals/genotype/sex and all tests were repeated with a second cohort of mice. Male and female mice were individually housed for at least 7 days before the beginning of behavioral testing. All testing was performed between 6:30 and 13:00. The following testing order was used: open field (three consecutive days) and elevated plus maze 3 days later. All behavior was scored manually by investigators blinded to the phenotype of the animal. Open Field Test: General locomotor activity was measured in a 42 × 42 cm cage using the Digiscan Activity Monitor (Accuscan Instruments). Activity was measured for 15 min for 3 consecutive days. Total distance traveled and time in the center of the open field were measured automatically by the Digiscan Software Program (Accuscan Instruments). Elevated Plus Maze: The EPM consisted of four arms (27 × 6 cm) arranged in a plus form, and elevated 50 cm above the floor. Two opposing arms were surrounded on three sides with 15-cm-high clear Plexiglas walls (closed arms) and the other arms were left open (open arms). Mice were placed in the center of the maze facing an open arm and behavior was monitored for 5 minutes.

**Blood Collection, RIA, Basal and Acute Stress Analysis.** Plasma was isolated as previously described (Burrows, Nakajima et al. 1998) and plasma corticosterone and ACTH levels were determined with MP Biomedicals kits (Orangeburg, N.Y.). For basal A.M. and P.M. hormone levels, blood was collected within 1-2 hours of lights on and 30-60 minutes before lights off. Stress studies were performed at least 7 days after behavior tests.

**Statistical Analysis.** All statistical analyses were performed by *t* tests and ANOVA. Two-factor ANOVA was performed on *in situ assays* for MR and 5HT-1a (genotype x sex), as well as on the stress corticosterone levels (genotype x time).

***Results***

**Generation of forebrain specific MR overexpressing mice**. To generate transgenic mice that overexpress MR specifically in the forebrain (MRov), we used the previously described CaMKIIα promoter (Mayford, Bach et al. 1996) to direct expression of a 3X Flag-tagged MR cDNA. The 3X Flag epitope tag was added at the N-terminus of MR to allow detection of transgene-directed MR (Figure 2-1A). The addition of the 3X Flag epitope did not significantly alter the transcriptional activation properties of MR (Figure 2-1B), and MR and Flag-MR showed similar transactivation profiles across corticosterone concentrations from 10 pM to 100 nM (data not shown). The binding properties of MR and Flag-MR expressed *in vitro* were also not significantly different (Figure 2-1C). The transgene construct was then utilized for production of transgenic mice.

*In situ* hybridization (ISH) analysis using both MR and transgene specific probes (delineated in Figure 2-2A) was used to determine the sites of transgene expression in 10 different founder lines. Of these 10 lines, only 7 expressed the transgene. Transgene-directed MR expression was localized largely to the hippocampus and cortex, with varying intensities of expression in these regions seen between the different lines (data not shown). As expression patterns were similar in most lines, lines 743 and 709 were

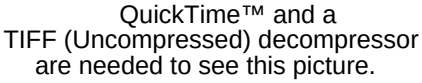


**Figure 2-1 Transgene construct and properties of Flag-MR.**

(A) Schematic of transgene construct. The CaMKIIα promoter directs expression of a 3X Flag-tagged mouse MR cDNA. Arrows represent the location of the primer pair used for PCR genotyping. The two bars represent the locations of the two probes used for *in situ* hybridization analysis (TG = transgene specific, MR = endogenous MR + transgene directed MR). NTD = N-terminal domain, DBD = DNA binding domain, and LBD = ligand binding domain. (B) Transactivation of GRE-Luciferase by MR vs. Flag-MR. CV-1 cells were transfected with MR, Flag-MR or vector alone, GRE-Luciferase, and CMV-βgal. 24 hours later cells were treated with drugs for an additional 24 hours (corticosterone = 100nM, aldosterone = 10nM). Normalized luciferase activity (luciferase activity/βgal activity) for samples was divided by their respective no drug control to result in fold induction of reporter gene activity. The data are presented as the mean ± S.E.M. and represent 3-5 independent experiments done in duplicate. (C) Ligand binding properties of MR vs. Flag-MR. Cos-1 cells were transfected with MR or Flag-MR. Cells were harvested after 40 hours and $^{3}H$ corticosterone was used to determine dissociation constants ($K_d$) for MR and Flag-MR in 5 independent Scatchard analyses. Data represent mean ± S.E.M.

selected for further analysis. ISH with sagittal sections demonstrated that MR transgene mRNA was detected specifically in forebrain regions (Figure 2-2A). RT-PCR experiments using RNA from brain and peripheral tissues revealed no transgene expression outside of the forebrain (data not shown). While transgene mRNA was widely expressed in cortex and all subfields of the hippocampus, ISH experiments with a transgene specific probe showed no expression of the transgene in either the PVN (Figure 2-3) or the amygdala of MRov mice, additional sites of endogenous MR expression (Arriza, Simerly et al. 1988; Ahima, Krozowski et al. 1991; Han, Ozawa et al. 2005). Western analysis using tissue prepared from different brain regions and a transgene specific anti-Flag antibody confirmed the presence of transgene directed Flag-MR protein in hippocampal and cortical regions for both lines (Figure 2-2B) consistent with *in situ* hybridization results. The very low levels of Flag-MR protein seen in the hypothalamus and hindbrain/cerebellum (HB) lanes for line 743 females were not observed in additional analyses, consistent with the lack of transgene specific mRNA in these regions.

To determine whether transgene expression in the hippocampus of MRov mice resulted in overexpression of total MR mRNA relative to wildtype littermates, ISH experiments using an MR specific probe that detects both endogenous and transgene directed MR were performed using both wildtype and transgenic male and female mice (Figure 2-2C, line 743). ANOVA analysis reveals a significant effect of genotype ($p < 0.05$) in all sub-regions of the hippocampus (CA1, CA2, CA3 and Dentate Gyrus (DG)) with MRov mice exhibiting greater levels of expression, and no gender effects in any of the sub-regions. If the data are analyzed separately to compare either male or female

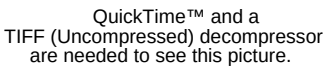


**Figure 2-2 Forebrain specific transgene expression in MRov mice.**

(A) ISH with MR and transgene-specific (TG) probes on sagittal sections from WT and two different MRov founder lines reveals forebrain specificity of transgene expression. (B) Western blot analysis with anti-Flag antibody confirms expression of transgene in forebrain regions of both male and female mice. Regions include hippocampus (Hip), hypothalamus (Hyp), cortex (Ctx), hindbrain/cerebellum (HB), and pituitary (Pit). (C) Representative pseudo-colored ISH images with an MR specific cRNA probe show overexpression of MR mRNA in sub-regions of the hippocampus (blue = low expression and red = high expression). Quantitation represents Integrated Optical Density (IOD) analysis of 4-5 animals/genotype/sex and 9-14 sections/animal. Data are presented as mean ± S.E.M. and * = $P < 0.05$ by t-test.

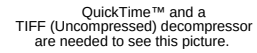


**Figure 2-3 Transgene expression is not found in the hypothalamus of MRov mice.**

ISH analyses with a transgene specific cRNA probe show clear expression of the transgene in the hippocampus (slides 34 and 35) and cortex, with no detectable expression of the transgene mRNA in the PVN. Cresyl violet stains of boxed regions indicate that slides 7, 5 and 10 contain the PVN (arrows).

transgenics with their respective wildtype littermates, a significantly higher expression of total MR mRNA in CA1, CA2, and CA3 ($p < 0.05$ for each sub-region) in female MRov mice was seen. In male transgenic mice, a significant increase in MR mRNA was seen only in CA2 ($p < 0.05$), with a trend towards increased MR mRNA in CA3 ($p = 0.06$). In a second set of ISH studies, where males and females were analyzed in separate experiments, similar results were obtained. The mean grey levels from this experiment (not corrected for area) (Figure 2-4) show that MR mRNA levels for transgenic and wildtype mice are greatest in CA2 and DG.

Together, these data demonstrate that transgenic MRov mice overexpress hippocampal MR mRNA by approximately 20-25% relative to wildtype littermate controls, representing a physiologically relevant increase in expression *in vivo*. Moreover, female mice tend to show more areas of hippocampal overexpression than males. As shown above, the MRov mice also express significant levels of MR in the cerebral cortex, a site of increased MR expression in primates, allowing potential functional interactions of MR alone or with GR in additional cortical sites.

**Normal basal HPA axis activity, but sexually dimorphic corticosterone release in response to restraint stress in MRov mice.** The traditional view of MR function in HPA axis control suggests a primary involvement in the maintenance of basal tone with little to no role in the response to stress. To assess the effects of forebrain MR overexpression on basal HPA axis activity, circadian trough and peak corticosterone levels were determined. Plasma samples from transgenic line 743 and wildtype littermates were collected 1-2 hours after lights on or 30-60 minutes before lights off.

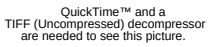


**Figure 2-4 Increased expression of MR mRNA in hippocampus of MRov mice.**

ISH analyses of a second cohort of female (A) and male (B) MRov mice from line 743 with an MR specific cRNA probe reveals increased levels of MR in a subregion-specific manner. Quantitation represents analyses of mean gray levels (instead of IOD) of 4-5 animals/genotype/sex and 6-12 sections/animal for females and 11-15 sections/animal for males. Data are presented as mean ± S.E.M. and *$P < 0.05$ by t-test.

There were no differences between transgenics and wildtype littermate controls for either sex at the nadir of the circadian rhythm (Figure 2-5A). In two independent experiments, corticosterone levels were 20-27% lower in both male and female transgenic mice at the peak of the circadian rhythm, however this did not reach significance (Figure 2-5B, experiment 1 shown, 20 and 22% reductions, respectively). Because all anxiety-like behavior tests constitute mild stressors, we also assessed corticosterone levels in MRov mice under mild stress conditions. Plasma samples were collected 10 minutes after a 5-minute exposure to the elevated plus maze; no significant differences between transgenics and wildtypes were observed for either sex (data not shown). To assess HPA axis function in response to more intense stressor, male and female transgenic and wildtype mice were subjected to 30 min. of restraint stress. Plasma samples were collected at 0, 20, 40, and 60 minutes (Figure 2-5C). There was no significant difference in corticosterone levels between male transgenic and wildtype mice. Female transgenic mice, however, exhibited a moderate suppression of the corticosterone response to stress throughout the stress and recovery timepoints compared to wild-type littermates ($p < 0.05$); no significant differences were observed in ACTH levels. These results demonstrate a sexually dimorphic corticosterone response to restraint stress in the MRov mice.

**MRov mice exhibit decreased anxiety-like behavior.** Previous studies using icv MR antagonist administration in rats suggest that temporary blockade of MR in the brain results in decreased anxiety (Korte, de Boer et al. 1995; Smythe, Murphy et al. 1997), suggesting that increased MR signaling would be anxiogenic. To further address the role

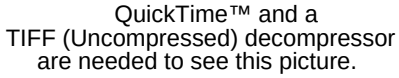


**Figure 2-5 Normal basal HPA axis activity but sexually dimorphic suppression of corticosterone release in response to restraint stress.**

Basal A.M. corticosterone levels (n = 4-5/genotype/sex). (B) basal P.M. corticosterone levels (n = 5-6/genotype/sex). (C) Restraint stress-induced corticosterone release in male and female MRov and wildtype mice in the A.M. Female MRov mice exhibit a moderate suppression of the corticosterone response to stress over the stress and recovery time course. ANOVA analysis reveals a significant difference due to genotype, $p = 0.029$ (n = 4-10/genotype/timepoint).

of constitutive forebrain MR overexpression in anxiety-like behavior, MRov mice were analyzed in a series of behavioral paradigms. These include general locomotor activity and time in the center of the open field, in addition to the elevated plus maze (EPM). Transgenic and wildtype mice of both sexes were initially tested for general locomotor activity for 15 minutes in the open field for 3 consecutive days. These data were collected in five 3-minute bins per day. As subsequent behavioral tests are 5 minute tests, only the first 2 bins (6 minutes) are presented. For line 743, the results show that MRov and wildtype mice both habituate normally to the open field and do not reveal any changes in general locomotor activity over the testing period (Figure 2-6A). Thus, forebrain MR overexpression does not result in any generalized locomotor alterations that could obscure further behavioral analyses.

To determine the consequences of forebrain overexpression of MR on anxiety-like behaviors, the time spent in the center of the open field was determined for male and female MRov and wildtype mice. MRov mice of both sexes exhibited increased time in center on days two and/or three of testing, an indicator of decreased anxiety-like behavior (Figure 2-6B). Male and female MRov mice and wildtype controls were also tested on the EPM, a widely used test of anxiety-like behavior. Male MRov mice spent a significantly greater percentage of time in open arms of the EPM compared to wild-type controls ($p < 0.05$, Figure 2-6C). The latency to first entry on the open arm was significantly less in male MRov mice compared to wt ($p < 0.05$). In an independent group of animals, significant differences were observed in these two measures in addition to showing a significant increase in percent open arm entries (data not shown). Over two rounds of testing, female MRov mice spent a significantly greater percentage of time in open

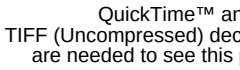


**Figure 2-6 Reduced anxiety-like behavior in forebrain MRov mice.**

(A) Three days of open field exposure show normal habituation and no changes in locomotor activity in either male or female line 743 MRov mice (first 6 minutes of activity shown). (B-C) Anxiety-like behavior measures in male and female MRov mice. (B) Open field activity reveals increased center time on days 2 and/or 3. (C) MRov mice show decreases in measures of anxiety-like behavior on the EPM, with significant changes in % Open Arm Time, % Open Arm Entries (females only), and Open Arm Latency. Data represent mean +/- S.E.M. of 19-40 animals/genotype/sex and * = $p < 0.05$.

arms of the EPM compared to wildtype controls ($p < 0.05$, Figure 2-6C). They also showed a significant increase in the percent of open arm entries ($p < 0.05$) and a significant decrease in the latency to the first entry on an open arm ($p = 0.05$).

To rule out the possibility of insertion site effects, most of these behavioral experiments were repeated in another line of MRov mice (line 709). While the phenotype was not as pronounced in line 709 as it was in line 743, there were many similarities, suggesting that the decreased anxiety-like behavior is not due to an insertion site effect (Figure 2-7).

**Basal expression of genes associated with HPA axis activity**. We hypothesized that the constitutive overexpression of MR in forebrain regions might result in altered expression of genes associated with HPA axis activity and anxiety-like behaviors. To address this hypothesis, the basal or stress-induced expression patterns of CRH and hnCRH in PVN, GR in hippocampus and PVN, and 5HT-1a in hippocampus were analyzed by *in situ* hybridization in MRov mice. Region CA1 of the hippocampus showed a significant *decrease* in the expression of GR mRNA in male MRov mice ($p < 0.05$), while in female MRov mice, this same region showed a non-significant trend towards decreased GR mRNA ($p = 0.11$, Figure 2-8A). This effect was selective to the hippocampus as no significant changes in GR mRNA levels were observed in the PVN (Table 2-1). There were also no significant changes in the expression of steady-state CRH mRNA levels in the PVN of male and female transgenic mice and no changes in hnCRH in the PVN of transgenic mice after 20 minutes of restraint stress for either sex (Table 2-1). Interestingly, both male and female MRov mice showed a significant *increase* in

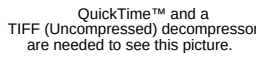


**Figure 2-7 Assessment of anxiety-like behavior on EPM in line 709 MRov female and male mice.**

Whereas line 709 expressed increased levels of Flag-MR compared to line 743 on immunoblot analaysis, *in situ* hybridization studies showed that total hippocampal MR mRNA in line 709 was increased over wild type (wt), but similar to or less than hippocampal MR mRNA levels in line 743. Endogenous MR expression was likely decreased in line 709 in response to increased Flag-MR as part of a compensatory mechanism, consistent with autologous regulation of MR expression (38). The modest increase in total MR mRNA in line 709 may explain why the behavioral changes are less dramatic in this line than in line 743. (A) Decreased anxiety-like behavior in line 709 female MRov mice with decreased open arm latency, and a trend towards increased % open arm time on the EPM. (B) Line 709 male MRov mice show nonsignificant changes in the same directions as line 709 female MRov mice. Data represent mean +/- S.E.M. of 18-28 animals/genotype/sex and *$p < 0.05$ and =$p = 0.06$.

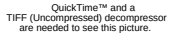




**Figure 2-8 Altered expression of GR and 5HT1a in MRov mice.**

(A) ISH analyses with a GR specific probe show a decrease in GR mRNA in region CA1 of the hippocampus of male MRov mice and a trend towards decreased GR in region CA1 of female MRov mice with n = 5 animals/genotype/sex, and 12-16 sections/animal for males and 7-9 sections/animal for females. (B) ISH with a 5HT-1a specific probe shows significantly increased levels of the receptor mRNA in the CA1 of male and female MRov mice with n = 4-5 animals/genotype/sex, and 9-12 sections/animal. * = $p < 0.05$, = = $p = 0.11$. (C) 5HT-1a receptor binding with [$^3$H]-8-OH-DPAT shows significantly increased levels of binding (** = $p = 0.0004$) with n = 6 males/genotype and 8-10 sections/animal. Representative pseudo-colored images are shown.

**Table 2-1 HPA axis gene expression profiles**

| | | |
|---|---|---|
| **Male** | | |
| **PVN** | **WT** | **MRov** |
| basal GR | 15,779 ± 2070 | 11,208 ± 1,470 |
| basal CRH | 24,921 ± 5,471 | 21,183 ± 4,274 |
| hnCRH | 38,157 ± 2,932 | 40,314 ± 1,279 |
| **Female** | | |
| **PVN** | | |
| basal GR | 23,048 ± 2,872 | 24,757 ± 1,683 |
| basal CRH | 27,911 ± 3,319 | 26,277 ± 2,108 |
| hnCRH | 38,591 ± 3,749 | 34,900 ± 4,095 |

GR, CRH, and stress induced hnCRH RNA expression in PVN of male and female MRov mice. Quantitation was performed as described in Materials and Methods. Data are presented as the mean ± S.E.M. in integrated optical density units obtained from ISH analyses. Male and female ISH results were obtained in independent experiments.

the expression of 5HT-1a mRNA in region CA1 of the hippocampus (Figure 2-8B, $p < 0.05$). The transgenic mice also showed an increase in [$^3$H]-8-OH-DPAT binding in CA1 (Figure 2-8C, WT = 0.71 and TG = 0.76, $p = 0.0004$), consistent with elevated levels of 5HT-1a protein. The relative change in 5HT-1a receptor binding was less than the change in mRNA levels, consistent with previous studies (Korte, Meijer et al. 1996). Together, these results suggest that overexpression of MR in the forebrain alters the basal mRNA expression patterns of GR and 5HT-1A, genes involved in both HPA axis function and anxiety-related behaviors.

***Discussion:***

In this study, we characterize anxiety-like behaviors and the HPA axis of transgenic mice with chronic forebrain-specific overexpression of MR. We show that chronic elevations of forebrain MR result in decreased anxiety-like behaviors in both male and female transgenic mice. We also demonstrate that increased levels of forebrain MR result in a moderate suppression of the stress response in female transgenic mice, suggesting that, at least in females, MR contributes to negative feedback information during a stressful event. Furthermore, MR overexpression alters mRNA levels of two genes associated with stress and anxiety, increasing hippocampal 5HT-1A and decreasing hippocampal GR expression. This latter change results in a significantly altered MR:GR ratio in certain areas of the hippocampus. Our findings suggest that the functions of forebrain MR overlap with GR in HPA axis regulation but counterbalance GR in modulation of anxiety related behaviors.

**Anxiety-like behavior.**

Both male and female MRov mice exhibit a decreased anxiety-like behavior phenotype. These data are at odds with a series of antagonist studies that suggest that MR activation plays an anxiogenic role either alone or in combination with GR. There are, however, two prominent changes at the molecular level in the MRov mice that may account for this result. First, forebrain GR plays an anxiogenic role in several genetic models of altered GR levels (Montkowski, Barden et al. 1995; Tronche, Kellendonk et al. 1999; Wei, Lu et al. 2004) and it is downregulated in certain hippocampal areas of the MRov mice, consistent with MR-mediated downregulation of GR (Herman and Spencer 1998). Thus, it is possible that overexpression of MR in the forebrain of MRov mice reduces anxiety behavior through a decrease in GR function, or through alterations of the GR/MR ratio which has been proposed to have functional import (De Kloet, Vreugdenhil et al. 1998). Second, decreased anxiety-like behavior in the MRov transgenic mice may result from the increased expression of 5HT-1a in CA1 of both male and female MRov mice. Multiple genetic manipulation studies of 5HT-1a suggest that this receptor plays an anxiolytic role, and that post-synaptic sites of 5HT-1a, such as the hippocampus, mediate the anxiolytic effects (Gordon and Hen 2004).

The increased expression of 5HT-1a receptor in the hippocampus of MRov mice was not predicted as 5HT-1a receptors are under tonic inhibition by corticosterone *in vivo* (Chalmers, Kwak et al. 1993), and studies by de Kloet (De Kloet, Sybesma et al. 1986) suggest that MR is the primary inhibitor of 5HT-1a expression. In addition, *in vitro* studies show direct repression of 5HT-1a promoter activity by binding of MR and GR as both homo- and heterodimers to a novel negative glucocorticoid responsive element

(nGRE) in the rat 5HT-1a promoter (Ou, Storring et al. 2001). Multiple hypotheses can be proposed to explain the observed increase in hippocampal 5HT-1a in MRov mice. First, while adrenalectomy studies are short term, MRov mice experience chronically elevated levels of MR for most of their lives. Hence, while the decrease in corticosterone levels seen at the PM time point in male and female MRov mice is not statistically significant, it may, over the course of the lives of the animals, represent a significant biological decrease in glucocorticoid signaling that allows for enhanced 5HT-1a expression in the hippocampus. There may, in addition, be a critical developmental time window during which MR overexpression may result in lifelong changes in serotonin receptor expression. Moreover, the change in hippocampal MR/GR ratio in MRov mice may affect the expression pattern of 5HT-1a through altered signaling on the 5HT-1a promoter directly or through an indirect mechanism such as changes in expression of other transcription factors. While the mechanism remains to be elucidated, it is evident that chronic MR overexpression results in downstream changes that have different molecular and behavioral outcomes from short-term alterations in MR signaling. Of note is that the change in MR expression is relatively subtle and may well occur in the context of normal variability in gene expression. That such a modest and localized change in gene expression can result in lifelong changes in responsiveness to affective stimuli underscores the role of MR, and its partner GR, in fine-tuning reactivity to environmental stimuli.

**HPA axis regulation:**

Several extra-hypothalamic sites have been shown to inhibit HPA axis activity in a corticosterone-dependent manner including the hippocampus (Kovacs and Makara 1988; van Haarst, Oitzl et al. 1997) and the cingulate cortex (Diorio, Viau et al. 1993), both of which express MR and GR. While it is clear that GR in these forebrain sites is important for HPA axis inhibition (Boyle, Brewer et al. 2005), the contribution of MR to HPA axis feedback post-stress or at the peak of the circadian rhythm is currently under debate. Female MRov mice, which have increased levels of MR compared to wt controls in cortex and in all sub-regions of the hippocampus do not exhibit significant alterations in basal corticosterone levels, or an altered endocrine response to mild stress. However, they do show a small but significant attenuation in their response to restraint stress.

These data suggest that as MR becomes progressively more occupied in female MRov mice, it is capable of inhibiting the stress response and to a lesser degree, the pm corticosterone levels. Recent data from Kalman et. al. (Kalman and Spencer 2002) support the potential biological relevance of this overexpression model. They find that MR occupation at the nadir of the circadian rhythm is closer to 50% rather than the previously thought 90%, suggesting a more dynamic capability of MR signaling in response to stress (Kalman and Spencer 2002).

The *in situ* hybridization data show that, relative to wildtype mice, female transgenic mice show a greater percent increase in MR mRNA than the male mice. This result suggests that a threshold mechanism may be in place, where the percent change in MR may be important for its relative contribution towards negative feedback during a

stressful event. This may account, in part, for the sexually dimorphic alteration in stress response observed in the MRov model.

**Comparison with MR knock-out models:**

In 1998, mice were generated that lack MR globally, but these mice die of dehydration by postnatal day 10 due to renal sodium and water loss, not allowing for behavioral analyses (Berger, Bleich et al. 1998). Very recently, a forebrain-specific MR-deficient mouse was created using the CaMKIIα promoter to direct cre recombinase activity only in forebrain regions ($MR^{CamKCre}$) (Berger, Wolfer et al. 2006). No alterations in corticosterone levels were seen either under basal AM or PM conditions or after 40 minutes of restraint stress. Also, contrary to the findings in MRov mice, no differences were seen in anxiety-like behaviors. There are, however, several significant differences between these two genetic models of increased or decreased forebrain MR. First, the respective mice are on slightly different genetic backgrounds. Second, while behavioral testing in MRov mice was performed during the nadir of the circadian rhythm (in the light period), all of the behavioral testing for the $MR^{CamKCre}$ mice was performed during the dark cycle when corticosterone levels are higher. This may result in a greater proportion of activated GR, while the testing in MRov mice is under predominant MR activation. Third, the loss of MR in $MR^{CamKCre}$ mice appears to be more extensive than the overexpression of MR in MRov mice. For example, MR is lost in the amygdala in $MR^{CamKCre}$ mice, but MR is not overexpressed in the amygdala of MRov mice. Fourth, the MRov mice exhibit increased expression of MR in cortical regions that normally express low or non-detectable levels of MR in mice. Combined, these differences may result in a

differential expression of compensatory mechanisms leading, overall, to altered patterns of behavior or HPA axis function in the $MR^{CamKCre}$ and MRov mice.

In conclusion, we have created a transgenic mouse with physiologically relevant increases in MR expression in the forebrain to assess the role of MR in anxiety-like behavior and HPA function. The data demonstrate a significant role for MR in the reduction of anxiety-related behaviors and suggest important roles for MR signaling in HPA axis activity beyond maintenance of basal tone. These data also highlight the complex nature of MR and GR signaling which may work together in the regulation of some pathways, but exhibit distinct activities in the modulation of others.

Acknowledgements:

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*Appendix 1*

***The Effects of Forebrain Overexpression of MR on Neurogenesis***

**Introduction:**

Corticosteroid production is tightly controlled by the Hypothalamic Pituitary Adrenal (HPA) axis. Throughout the circadian rhythm and in response to stress, corticosteroids are released into the blood stream and serve to alter metabolic processes in a manner that allows the organism to adapt. While short-term activation of the pathway is adaptive, long-term elevations in corticosteroid levels have been shown to result in deleterious consequences, such as loss of hippocampal volume, dendritic tree retraction (Woolley, Gould et al. 1990), and decreased neurogenesis in the dentate gyrus of the hippocampus. Hypersecretion of corticosterone has been linked to multiple mood disorders including major depression and anxiety, and it is hypothesized that the structural remodeling of the hippocampus accompanying glucocorticoid hypersecretion may contribute to or exacerbate these pathophysiologies (McEwen 2005).

Corticosteroid action is mediated via two types of receptors, the high affinity mineralocorticoid receptor (MR) ($K_d$ corticosterone = 0.5nM) and the lower affinity glucocorticoid receptor (GR) ($K_d$ corticosterone = 5nM). MR and GR are both members of the steroid/thyroid hormone receptor superfamily that function as ligand-activated transcription factors. MR and GR normally reside in the cytoplasm, dimerize upon ligand binding and translocate to the nucleus where they are capable of regulating the transcription of genes whose promoters contain a glucocorticoid responsive element (GRE). Their different affinities for corticosterone suggest that they are activated

differentially over the course of the circadian rhythm and during times of stress. GR expression is widely distributed throughout the brain, whereas MR, while present in multiple regions, is most highly expressed in the hippocampus. In regions where MR and GR are coexpressed, such as the hippocampus, they can homodimerize as well as heterodimerize, allowing for increased complexity in corticosteroid signaling.

While MR and GR are both activated by corticosterone, the cellular consequences of their activation can often oppose each other, even in the same cell. For example, MR activation results in increased long-term potentiation (LTP) while GR activation decreases LTP (Pavlides, Watanabe et al. 1995). Similarly, MR activation can prevent the adrenalectomy-induced apoptosis in the dentate gyrus, whereas GR activation has been shown to have no effect or increase this apoptosis (Hornsby, Grootendorst et al. 1996). Because of the significant role that corticosteroids play in the structural remodeling of the hippocampus, and the potential importance of this remodeling in depression and anxiety, it is important to understand the individual roles of MR and GR in hippocampal remodeling events such as neurogenesis.

New neurons are continually being born in the mammalian dentate gyrus. The process of neurogenesis has been shown to be highly regulated by corticosterone. Rats treated acutely with corticosterone showed a significant decrease in the number of newborn neurons, while ADX results in a large increase in neurogenesis (Cameron and Gould 1994). Additionally, physiological alterations in corticosterone levels, such as acute and chronic stress are known to suppress hippocampal neurogenesis (Gould and Tanapat 1999). The activation of GR is clearly implicated in the suppression of neurogenesis in the dentate gyrus. High levels of corticosterone treatment given to either

adrenalectomized (Wong and Herbert 2005) or adrenal intact (Mayer, Klumpers et al. 2006) rats resulted in a suppression of proliferation that was normalized by antagonism of GR. Other evidence suggests that the GR agonist dexamethasone can inhibit neurogenesis both *in vivo* and *in vitro* (Kim, Ju et al. 2004). The effects of MR activation on neurogenesis are not as clear. Several groups have shown that MR activation can suppress the increased proliferation that accompanies adrenalectomy (Wong and Herbert 2005; Krugers, van der Linden et al. 2007) in a manner similar to GR. In contrast, however, it has also been shown that aldosterone can further enhance the increased proliferation that accompanies adrenalectomy (Fischer, von Rosenstiel et al. 2002). In support of this positive effect of MR activation on neurogenesis, genetic disruption of MR leads to impaired neurogenesis in the hippocampus, whereas brain specific disruption of GR had no effects on hippocampal neurogenesis (Gass, Kretz et al. 2000). In a series of *in vitro* experiments, primary hippocampal cultures treated with aldosterone showed enhanced neurogenesis that could be blocked with the MR antagonist spironolactone. In contrast, cultures treated with dexamethasone showed decreased neurogenesis, an effect that could be blocked by mifepristone (Fujioka, Fujioka et al. 2006).

In this study we sought to further address the role of MR in neurogenesis in a mouse model of forebrain MR overexpression previously characterized in our laboratory. These animals have been shown to overexpress MR in the hippocampus by 20-25%. Under basal conditions, MRov mice do not have any overt alterations in plasma corticosterone concentrations allowing for the analysis of basal levels of neurogenesis under a primary increase in MR signaling. Two well-described methods were used to detect newborn cells in the dentate gyrus: 1) BrdU incorporation into actively dividing

precursor cells; and 2) Ki67 staining that serves as a marker for cells in all stages of the cell cycle except $G_0$ (Wojtowicz and Kee 2006; Mandyam, Harburg et al. 2007).

**Materials and Methods:**

**Generation of MRov mice and Animal Husbandry.** A detailed description of the generation of the MRov mice is provided in a previous publication (Rozeboom, Akil et al. 2007). Mice were maintained on a 14:10 light/dark schedule with lights on between 6:00 and 20:00. Mice were given food (5008 mouse chow, 9% fat content; Purina Mills, Inc., St. Louis, MO) and water ad libitum. C57BL/6J (The Jackson Laboratory, Bar Harbor, ME) mice were bred at the University of Michigan. All mice in this study were 4–5 months of age. All procedures using mice were approved by the University of Michigan Committee on Use and Care of Animals. All experiments were conducted in accordance with the principles and procedures outlined in the National Institutes of Health guidelines for the Care and Use of Animals.

**Bromodeoxyuridine Administration.** Bromodeoxyuridine (BrdU, Sigma) was dissolved in normal saline (0.9% NaCl, .007M NaOH) at a concentration of 20mg/ml and filtered at 0.2μm. To label dividing cells, mice received 3 i.p. injections of BrdU (100 μg/g of body weight) spaced 4 hours apart (8am, 12pm, and 4pm). Mice were sacrificed 24 hours following the last injection (4pm).

**Tissue Processing.** Under anesthesia with sodium pentobarbital (50mg/kg), mice were transcardially perfused with 0.9% cold saline followed by cold 4% paraformaldehyde in

0.1M sodium phosphate buffer. Brains were removed from the animals and post-fixed for 1-2 hours at 4°C in the same fixative. Brains were then cryoprotected in a 20% sucrose/PBS solution overnight at 4°C. Brains were then frozen at -50°C in 2-methyl-butane and stored at -80°C until use. Cryostat sections through the dorsal hippocampus were cut at 30μm in the coronal plane. Five 1-in-5 series were collected in PBS for immediate use or transferred to a cryprotectant solution (30% ethylene glycol, 20% glycerol, and 50mM sodium phosphate buffer, pH 7.4) and stored at -20°C until use.

**Immunohistochemistry.** Detection of the proliferation marker Ki67 was performed as follows. Free-floating sections were mounted onto Superfrost plus (Fisher Scientific) slides and allowed to dry for several hours at room temperature. Sections were then washed in Tris-buffered saline (TBS) + 0.05% triton X-100 (TBST) on a shaker. Antigen retrieval was accomplished by heating the sections in 10% NaCitrate buffer for 40 minutes at 90°C. Sections were then allowed to cool to room temperature in the same buffer for 20 minutes and then endogenous peroxidase activity was quenched by incubating sections for 30 minutes in TBS + 0.001% $H_20_2$. Sections were subsequently washed in TBST, and blocked for 1 hour in BSA diluent (0.15M NaCl, 25mM $K_2HPO_4$, 17mM $KH_2PO_4$, 1% BSA, 1% normal goat serum, 0.4% Triton X-100). Sections were incubated with Ki67 antibody (provided by S.J. Watson laboratory) at 1:20,000 in BSA diluent overnight at room temperature (RT) with shaking. Sections were then washed in TBST, incubated for 1 hour at RT with a biotinylated goat anti-rabbit antibody at 1:1000 (Sigma, St. Louis, MO). Sections were washed in TBST and incubated for 80 minutes in Ready To Use (R.T.U.) (Vector). Sections were washed in TBST and incubated for 12

minutes in 3-3' diaminobenzidine (DAB) Tabs (Sigma) dissolved in water. Sections were rinsed in water, counterstained with cresyl violet, dehydrated through alcohols (50-100%), incubated in xylene 2-3 minutes and cover-slipped with Permount.

Immunohistochemistry for BrdU was performed as follows. Free-floating sections were transferred from cryoprotectant solution to TBS (0.5M NaCl, 0.1M Tris pH 7.5) and washed in several rinses for at least 3 hours. Endogenous peroxidase activity was quenched by incubation of sections in 0.1% $H_2O_2$ for 10 min. Antigen retrieval was achieved by incubation of sections for 2 hours at 65°C in 50% formamide/2X SSC, followed by 30 minutes in 2N HCl at 37°C and a 10 min. incubation in 100mM Na Borate, pH 8.5. Sections were blocked in BSA diluent as above, followed by overnight incubation of sections with 1:500 rat anti-BrdU (Accurate Chemical, Westbury, NY) at room temperature. Sections were washed in TBST and incubated for 1 hour at RT with a biotinylated goat anti-rat antibody at 1:1000 (Sigma). Sections were washed in TBST and incubated for 60 minutes in avidin-biotin complex (ABC) reagent (Vector) diluted 1:1000 in BSA diluent. Sections were then washed in TBST, incubated for 6 minutes in DAB (DAB tablets, Sigma), and washed in DI water. Sections were mounted onto slides, air dried, counterstained with cresyl violet, dehydrated through alcohols (50-100%), and coverslipped as above.

**Cell Counting and Dentate Gyrus Volume Estimates.** Cell counts were performed by an observer blinded to the genotype of the animal. A complete 1-in-5 series of sections through the dorsal dentate gyrus was analyzed for each animal. BrdU and Ki67 positive cells were counted in the subgranular layer of the dentate gyrus using a light microscope

at 63x magnification. Labelled cells in the subgranular layer (SGL), or ± 2 cell diameters of the SGL, and in all focal planes through the 30μm section were counted. The total number of BrdU and Ki67 positive cells was estimated by multiplying the number of labeled cells counted by 5. The volume of the granule cell layer was estimated for each animal in the same series of sections (analyzed at 10x magnification) by the method of Cavaliere using Stereo Investigator software (Williston, VT). The average labeled cell counts and volumes were determined for each group and subjected to unpaired t-test using Statview software.

**Results:**

The proliferation of dentate gyrus granule cells in wildtype and MRov mice was determined by immunohistochemical analysis for the incorporation of BrdU and the cell cycle marker Ki67. BrdU labeling was visible throughout the rostral-caudal extent of the dorsal dentate gyrus. Morphologically, labeled cells were irregularly shaped with DAB staining appearing as both multipunctate and densely uniform throughout the nucleus (Figure 2-9A). BrdU-labeled cells were most commonly found in clusters of 2 or more. Under basal conditions, there was no difference in the number of BrdU labeled cells between MRov and wildtype mice (Figure 2-9B).

Ki67 staining was also visible throughout the rostral-caudal extent of the dorsal hippocampus. Overall, the number of cells labeled for Ki67 was greater than BrdU labeled cells. This is most likely due to the fact that, while BrdU is only incorporated during S-phase, Ki67 is expressed during all stages of the cell cycle except $G_0$, suggesting that a larger population of cells may be available for labeling at any given time.

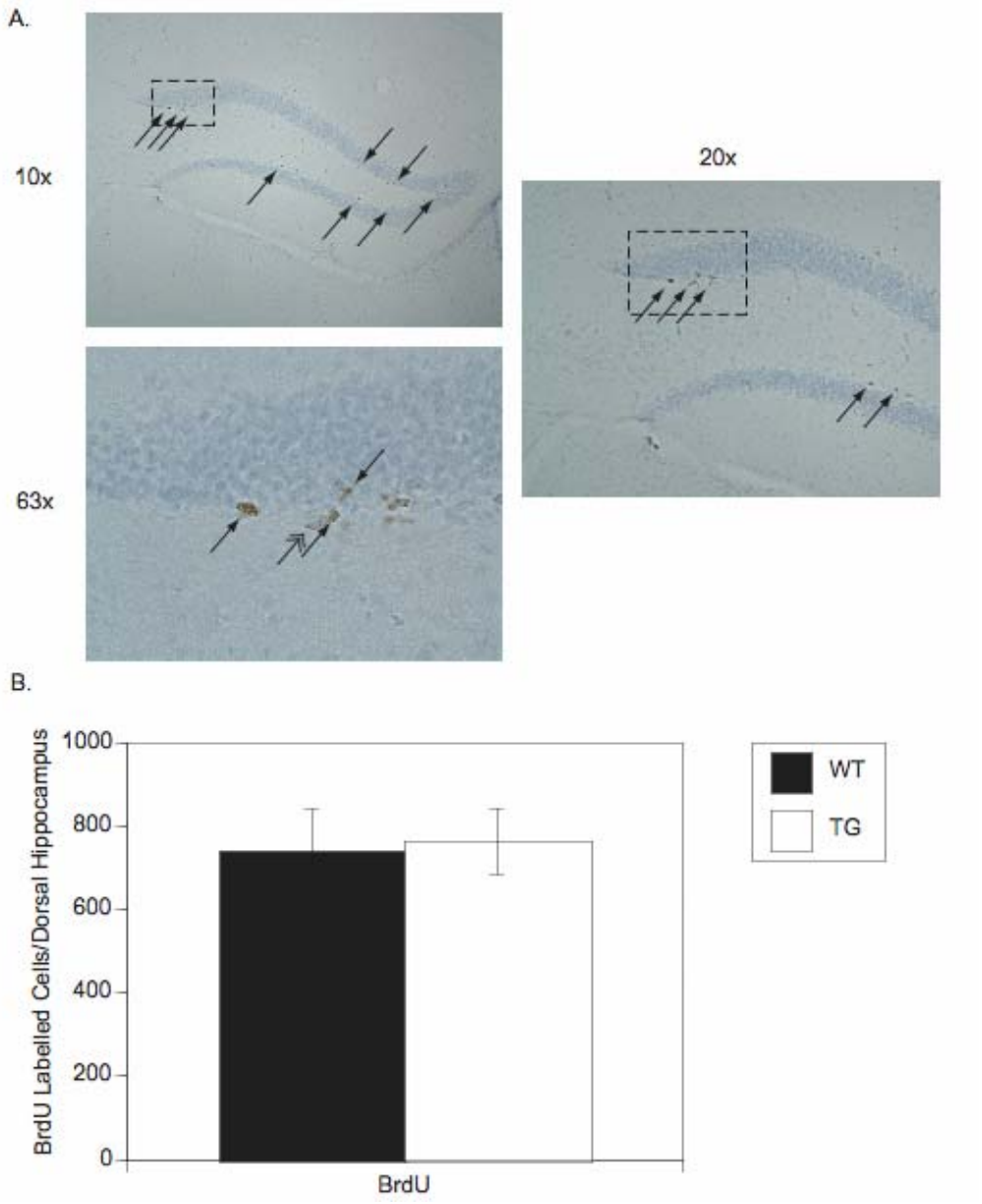



**Figure 2-9 Immunohistochemistry for BrdU in the dentate gyrus of the hippocampus.**

(A) The 10x magnification reveals BrdU positively stained cells (arrows denote examples of some of these cells) throughout the SGL of the upper and lower blades of the dentate gyrus. The 63x magnification reveals both densely (arrows) and diffusely (double arrowhead) stained clumps of cells in the SGL of the upper blade of the dentate gyrus. Dashed boxes denote area of enlargement. (B) Quantitation of BrdU labeled profiles throughout the rostral-caudal extent of the dorsal hippocampus of both wildtype (WT) and MRov (TG) mice.

Consistent with the BrdU data, there was no statistically significant difference in the number of Ki67 labeled cells between MRov and wildtype mice (Figure 2-10).

Any alterations in either proliferation or survival of newborn neurons in the dentate gyrus may result in differences in the size of this structure. To determine if the relative sizes of the dentate gyrus differed between MRov transgenic and wildtype mice, the Cavaliere method was used to estimate volumes in the same series of sections used to analyze Ki67 expression. Consistent with the neurogenesis data, there was no difference in mean volume estimates of the dentate gyrus between transgenic and wildtype mice (Figure 2-11).

**Discussion:**

Glucocorticoids have powerful effects on the proliferation and survival of newborn cells in the dentate gyrus. While the preponderance of data suggests that GR negatively regulates neurogenesis in the dentate gyrus, the role of MR in this process is not well understood. The goal of this study was to further delineate the role of MR in the regulation of proliferation in the dentate gyrus under basal conditions. To do this, we assessed the levels of neurogenesis in the previously characterized forebrain specific MR overexpressing mouse (Rozeboom, Akil et al. 2007) using two common methods for the determination of proliferation in the dentate gyrus: 1) BrdU incorporation by actively dividing precursor cells and 2) Ki67 staining that serves as a marker for cells in all stages of the cell cycle except $G_0$. Under baseline conditions, at 4-5 months of age, there were no differences in the levels of neurogenesis in the dorsal dentate gyrus between MRov and wildtype mice. Correspondingly, there were also no differences in the estimated

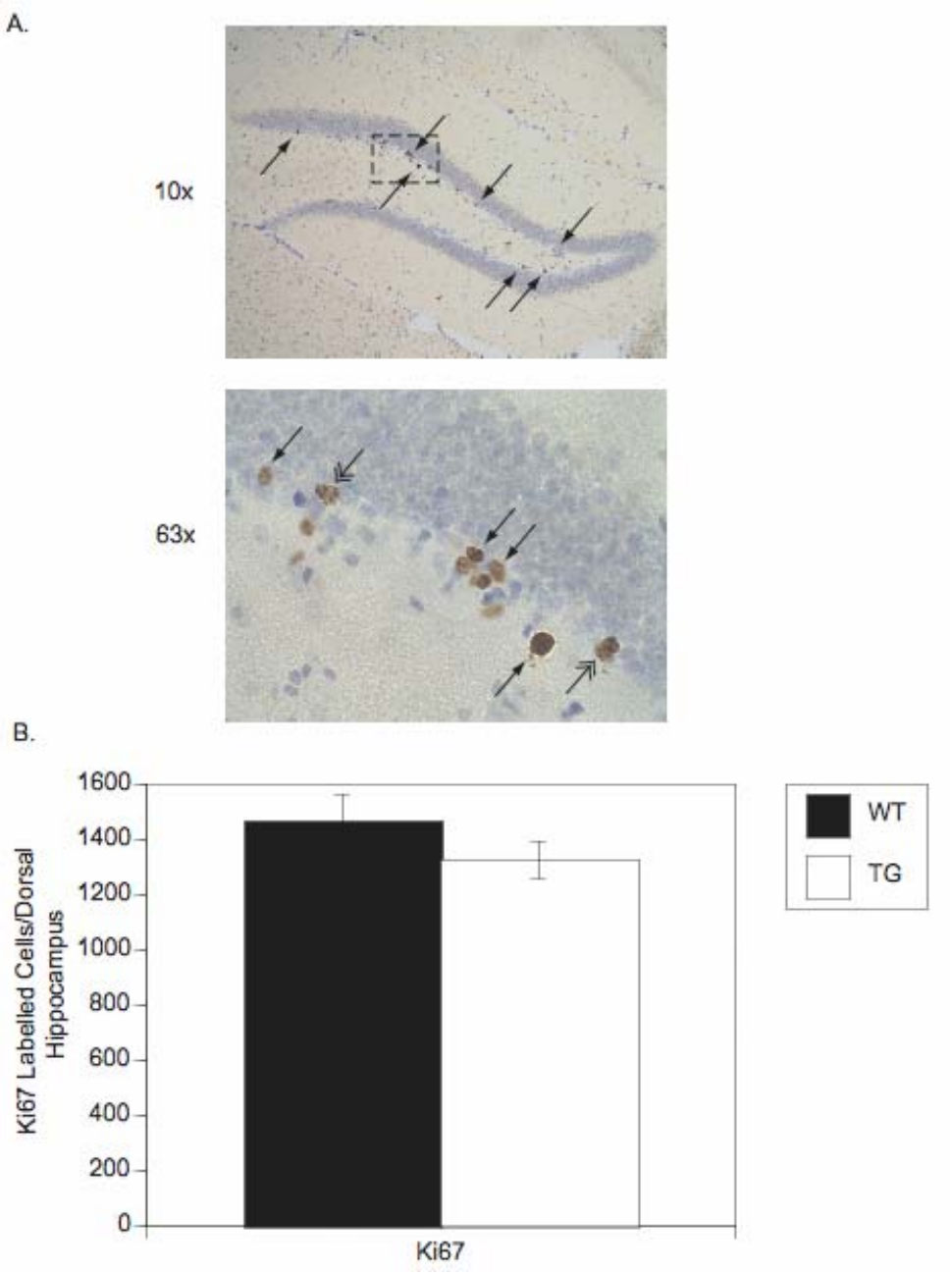



**Figure 2-10 Immunohistochemistry for Ki67 labeled cells in the dentate gyrus of the hippocampus.**

(A)The 10x magnification reveals Ki67 positively stained cells (arrows denote examples of some of these cells) throughout the SGL of the upper and lower blades of the dentate gyrus. The 63x magnification reveals both densely (arrows) and multipunctate (double arrowheads) stained clumps of cells in the SGL of the upper blade of the dentate gyrus. Dashed boxes denote area of enlargement. (B) Quantitation of Ki67 labeled profiles throughout the rostral-caudal extent of the dorsal hippocampus of both wildtype (WT) and MRov (TG) mice.

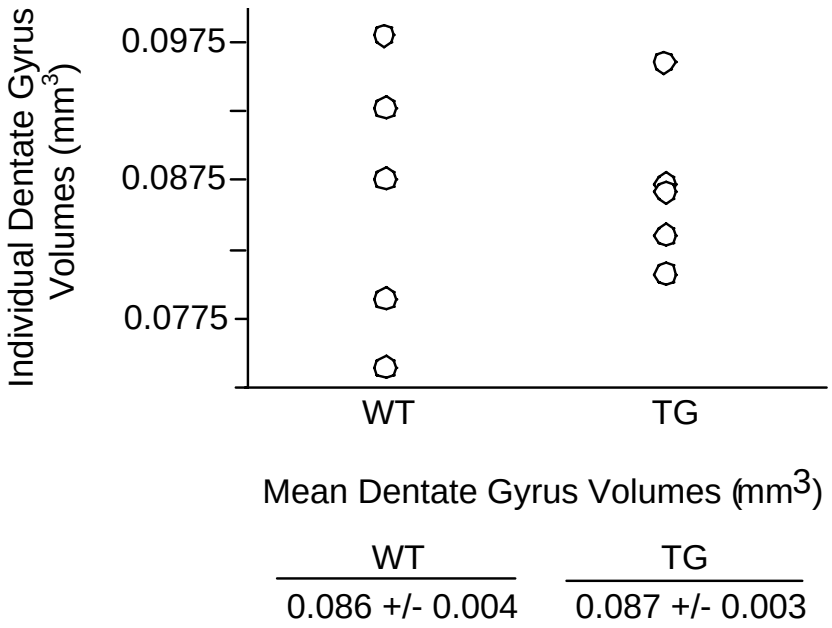



**Figure 2-11 Estimated volumes of the dorsal hippocampus.**

The Cavaliere method was used to estimate the volumes of the dentate gyrus in wildtype (WT) and MRov (TG) mice. The graph depicts the range of volume estimates for both groups. The mean volume estimates ± S.E.M. are listed below.

volumes of the dorsal dentate gyrus.

While previous data suggest that MR signaling is involved in the process of neurogenesis, there is currently no consensus concerning its exact role. Some studies suggest that MR activation can downregulate neurogenesis in a manner similar to GR (Wong and Herbert 2005; Krugers, van der Linden et al. 2007), while others suggest that MR activation can increase neurogenesis (Gass, Kretz et al. 2000; Fischer, von Rosenstiel et al. 2002). These experiments must be compared carefully, as there are both fundamental differences between paradigms used as well as differences in the manner in which studies are conducted within paradigms. For example, adrenalectomy and hormone replacement (Fischer, von Rosenstiel et al. 2002; Wong and Herbert 2005; Krugers, van der Linden et al. 2007) is useful for targeting MR activation directly. However, this paradigm only provides the opportunity to address short-term alterations in MR signaling and does not allow for the assessment of long-term alterations in signaling that may accompany a disease state. Additionally, methodological differences between studies such as the ligand used (aldosterone vs. corticosterone), dose of the ligand, and the route of ligand administration (injections vs. in the drinking water) make it difficult to directly compare the different studies and may help explain the variable results.

Conversely, while genetic manipulation studies allow for assessment of long-term changes in gene expression, genetically modified animals often exhibit compensatory changes in the system that could obfuscate the ability to study the specific pathway of interest. For example, MR knockout mice (MRKO) showed a decrease in neurogenesis in the dentate gyrus of the hippocampus (Gass, Kretz et al. 2000). A simultaneous comparison with mice containing a disruption of brain specific GR showed no changes in

neurogenesis, suggesting that the decreased neurogenesis in MR knockout mice is specific to the loss of MR. A complication in this interpretation arises when considering the compensatory changes that accompany the complete loss of MR in the organism. These mice normally die within a week of birth due to sodium imbalance, pointing to the important role of MR in sodium homeostasis through its activation by aldosterone in the kidney. MRKO mice can be rescued, however, through daily exogenous salt supplementation. While this allows the mice to survive to adulthood, it was found that the MRKO mice have elevated levels of corticosterone, perhaps due to the combination of the daily handling required for salt supplementation as well as loss of negative feedback provided to the HPA axis from MR. The increased levels of plasma corticosterone may therefore be responsible for the decreased neurogenesis through increased signaling at GR rather than a specific effect of decreased MR on neurogenesis.

In the forebrain of the MRov mice used in this study, there is a 20% elevation in hippocampal MR levels for most of the animals' lives. There are also no overt changes in baseline corticosteroid levels and no changes in the expression levels of GR in the dentate gyrus, allowing for the assessment of basal levels of neurogenesis under a primary increase in MR signaling without the complication of increased corticosterone levels or altered GR signaling. The fact that there are no changes in basal levels of neurogenesis in MRov mice may be explained by multiple possibilities. First, the ratio of hippocampal MR to GR expression is thought to have functional importance (De Kloet, Vreugdenhil et al. 1998). Thus, while the level of MR overexpression in these mice alters anxiety related behavior (Rozeboom, Akil et al. 2007), it may not be large enough to significantly alter neurogenesis. Alternatively, only a small proportion of progenitor

cells actually express GR, and none express MR (Garcia, Steiner et al. 2004) suggesting that the effects of corticosterone on proliferation are via an indirect mechanism that involves cell to cell communication. There are multiple cell types in the hippocampus that express MR and GR, including both neurons and glia, but it is currently unclear which populations of cells may be involved in this signaling (Bohn, Howard et al. 1991). In MRov mice, the CamKIIα promoter is used to drive expression of MR. This promoter is known to be neuron specific and largely glutamatergic (Liu and Jones 1996); hence it is possible that the overexpression of MR is limited to cell types not involved in the majority of signaling to precursor cells of the dentate gyrus.

The analysis of neurogenesis in this experiment was in young animals (4-5 months) under baseline conditions. There is evidence supporting the idea that under conditions of sustained increase in corticosteroids, such as chronic treatment with corticosterone, there is a decrease in the level of neurogenesis in the dentate gyrus that can be brought back to baseline levels through the blockade of GR alone (Mayer, Klumpers et al. 2006). Basal levels of corticosteroids are also elevated in aged rodents (Sapolsky 1985) and this correlates with a decrease in neurogenesis in old rats (Kuhn, Dickinson-Anson et al. 1996; Cameron and McKay 1999). Consistent with the idea that corticosteroids are involved in this decreased neurogenesis, removal of endogenous corticosteroids by adrenalectomy in young and aged rats increased the rate of cell proliferation to similar levels (Cameron and McKay 1999). Interestingly, studies have shown that MR levels are decreased under conditions of chronic stress and during the aging process in rats (Lopez, Chalmers et al. 1998; Hibberd, Yau et al. 2000). If the level of MR overexpression is too low to affect neurogenesis in young mice under baseline

conditions, it may be possible that the sustained increase in MR levels in MRov mice may prevent or attenuate the decreases in neurogenesis that accompanies chronic stress or aging. Future experiments will address these possibilities.

*Appendix 2*

***The effects of overexpressionof MR in the forebrain on learning and memory***

**Introduction:**

Glucocorticoid hormones are known to affect processes underlying learning and memory. On a cellular level, concentrations of glucocorticoids equivalent to a stressor decrease long-term potentiation (LTP), whereas low levels of glucocorticoids enhance LTP. Behaviorally, lower levels of glucocorticoids commensurate with low to moderate stress have been shown to enhance cognition in several learning and memory paradigms, whereas more severe stressors and higher levels of glucocorticoids disrupt it. While previous studies suggest that activation of MR and GR is clearly involved in cognitive function, differences in testing paradigms has made it difficult to draw overall conclusions concerning the exact roles of MR and GR. Short-term blockade of MR and GR suggests differential roles for MR and GR, affecting search escape strategies and processes underlying consolidation respectively. Long-term blockade of MR resulted in impaired learning and memory, whereas long-term blockade of GR, in contrast to acute effects, was shown to enhance learning and memory (Oitzl, Fluttert et al. 1998).

Recent studies addressing the affects of long-term alterations in GR and MR signaling through genetic modifications suggest that overexpression of GR in the forebrain results in mild cognitive impairments (Wei, Hebda-Bauer et al. 2007) and forebrain removal of MR results in impairments in learning and memory (Berger, Wolfer et al. 2006). Concurrent with studies in this thesis, a recent paper addressed the effects of overexpression of MR in the forebrain on learning and memory and observed mild

cognitive improvements (Lai, Horsburgh et al. 2007). As MRov mice also overexpress MR in the hippocampus, we were interested in determining the effects of increased MR in hippocampal-dependent learning and memory as well as retrieval of long-term memory through the use of long-term probe trials in the Morris Water Maze (MWM).

**Materials and Methods:**

**Animals.** MRov mice were bred and housed as previously described (Rozeboom, Akil et al. 2007). Four to 5 month old, gender matched, male and female MRov and wildtype mice were used in the Morris Water Maze.

**Morris Water Maze.** The Morris water maze (MWM) used in these experiments consisted of a 1.2-meter diameter pool filled with water that was made opaque with white nontoxic paint. Water temperature was maintained at 24 ±1 C° throughout the experiment. Every training trial began with the mouse on the platform for 15 sec. The mouse was then placed into the water facing the wall of the pool and allowed to search for the platform, with the starting position chosen pseudorandomly among six start positions. The trial ended either when the mouse climbed onto the platform or when 60 sec had elapsed. At the end of each trial, the mouse was allowed to rest on the platform for 15 sec. Mice were given 4 trials per day in blocks of 2 trials with inter-block times of approximately 90 minutes. Mice were trained for a total of 9 days. Probe trials were conducted 24 hours after every third day of training to monitor whether the mice were learning the task differentially. During the probe trial, the escape platform was removed and mice were placed in the pool at the start location directly opposite of the platform and

allowed to swim for 60 sec. If more training was to occur (days 4, 7, and 10), then the training took place directly after the probe trial. Long-term memory was assessed with probe trials performed 2.5 and 5 weeks after the final probe trial, with no further training between probe trials.

**Data Analysis.** Data were analysed by two-way repeated measures ANOVA with a repeated measure for training day.

**Results:**

Both MRov and wildtype (WT) mice were trained for 4 trials a day for 9 days on the hidden platform version of the Morris Water Maze. Acquisition data are shown in Figure 2-12. In both groups, the latency to reach the platform decreased as training progressed. There was a strong main effect of training day on the escape latencies for both groups ($F_{(8,152)} = 15.92$, $p < 0.0001$), however, there was no main effect of genotype on latency ($F_{(1,19)} = 0.229$, $p = 0.64$) and no interaction between training day and genotype ($F_{(8,152)} = 0..44$, $p = 0.9$). These data suggest that MRov and WT mice acquire the hidden platform equally well. In addition to measuring latency to the platform during training, probe trials were conducted 24 hours after the completion of training on days 3, 6, and 9. During probe trial 1, 24 hours after the third day of training, the amount of time each group spent in the Target quadrant (where the platform used to be) was not significantly greater than chance (25%) (Figure 2-13). During probe trial 2, 24 hours after the sixth day of training, while the WT mice spent significantly more time in the Target quadrant (40.4% ± 5.1%, $p < 0.05$, single group *t*-test compared to 25%), the MRov mice did not spend more time in the Target quadrant (29.0% ± 5.3%, $p = 0.47$). However, there

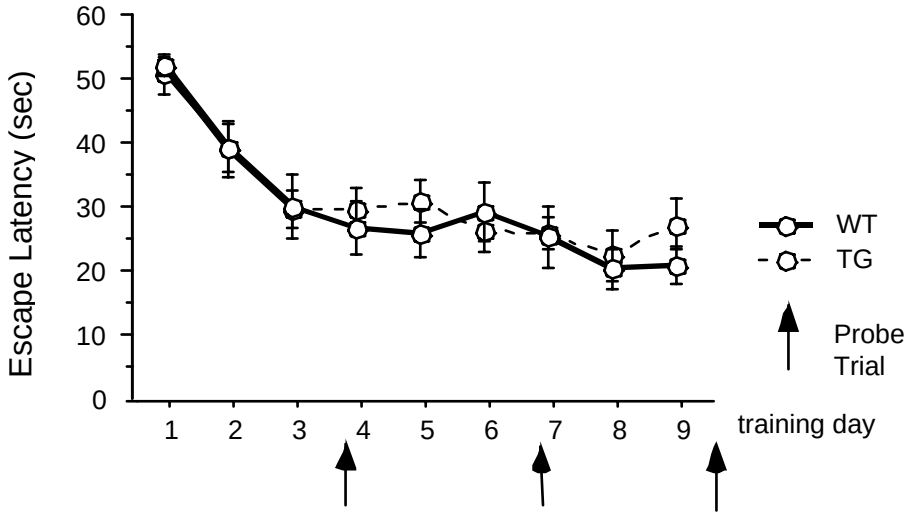



**Figure 2-12 MRov mice are not impaired in the acquisition phase of the Morris water maze.**

Mice were trained for 4 trials a day for 9 days. Time to reach the hidden platform (escape latency) was not significantly different for MRov (TG) mice when compared with WT littermate control mice.

was no significant difference in the amount of time that the MRov mice spent in the Target quadrant compared to their WT littermates ($p > 0.05$, unpaired *t*-test) (Figure 2-13). During probe trial 3, 24 hours after the final day of training, both MRov and WT mice spent significantly more time in the Target quadrant (MRov, 45.4% ± 5.9%, $p < 0.05$, and WT, 46.9% ± 4.7%, $p < 0.05$ single group *t*-test compared to 25%), and there was no difference between MRov and WT mice ($p > 0.05$, unpaired *t*-test).

To determine if overexpression of MR had an effect on long-term memory, probe trials were conducted 2.5 and 5 weeks following the final probe trial (Figure 2-14). During the first long-term probe trial, 2.5 weeks following the final probe trial, both MRov and WT mice spent significantly more time in the Target quadrant relative to chance (MRov, 42.7% ± 4.7%, $p < 0.05$, and WT, 36.7% ±4.5%, $p < 0.05$ single group *t*-test compared to 25%), and there was no difference between MRov and WT mice in the amount of time they spent in the Target quadrant ($p > 0.05$, unpaired *t*-test). During the second long-term probe trial, 5 weeks after the last probe trial, MRov mice spent significantly more time in the Target quadrant (47.9% ± 4.6%, $p < 0.05$ single group *t*-test compared to 25%), but WT mice did not (34.3% ± 5.0%, $p > 0.05$ single group *t*-test compared to 25%). While there was no significant difference between the MRov and WT mice in the amount of time that they each spent in the Target quadrant, there was a strong trend towards increased time by MRov mice relative to WT mice ($p = 0.06$).

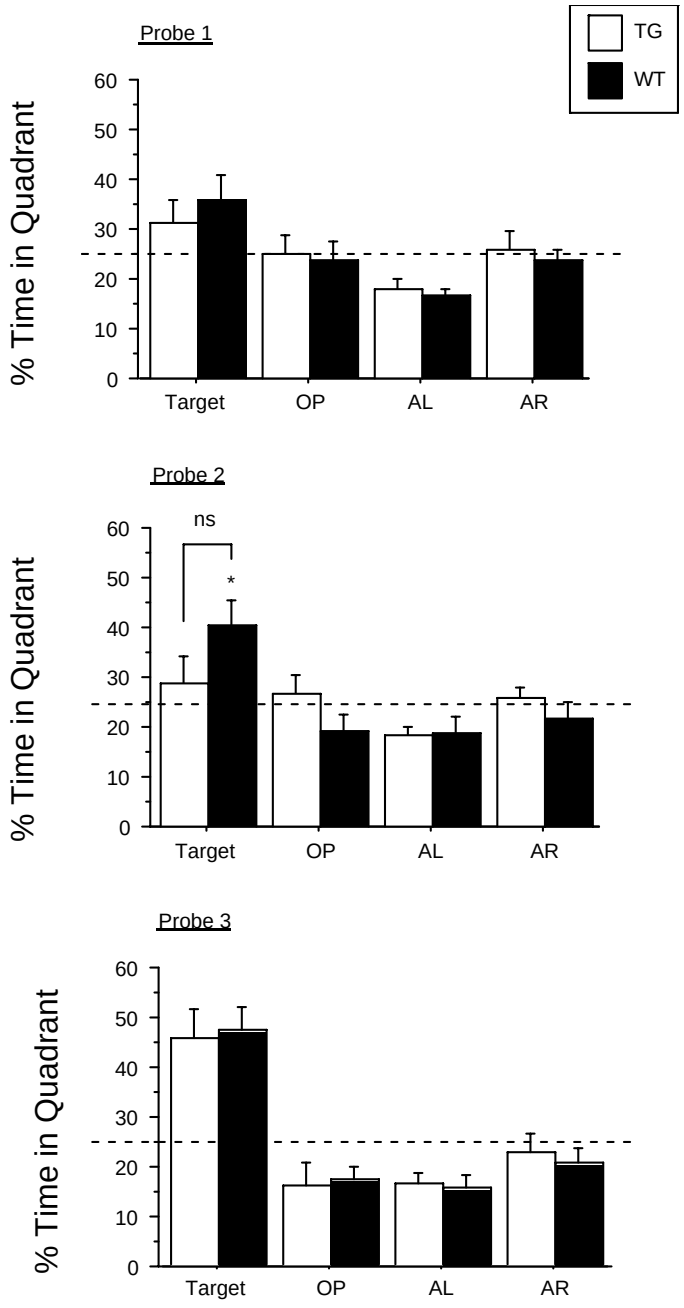



**Figure 2-13 Spatial memory is not altered in MRov mice**

A 60-second probe trial was completed 24 hours after every third day of training. During probe trial 1, neither group spent significantly more time in the quadrant where the platform used to be (Target). During probe trial 2, while WT mice spent more time in the Target quadrant and MRov (TG) mice did not, there was no significant difference between the groups. By the probe trial 3, both groups spent significantly more time in the Target quadrant. The dashed line (25%) represents random, or "chance" performance. (OP) Opposite; (AL) adjacent left; and (AR) adjacent right quadrants. *= $p < 0.05$ relative to 25% chance performance. NS = not significant.

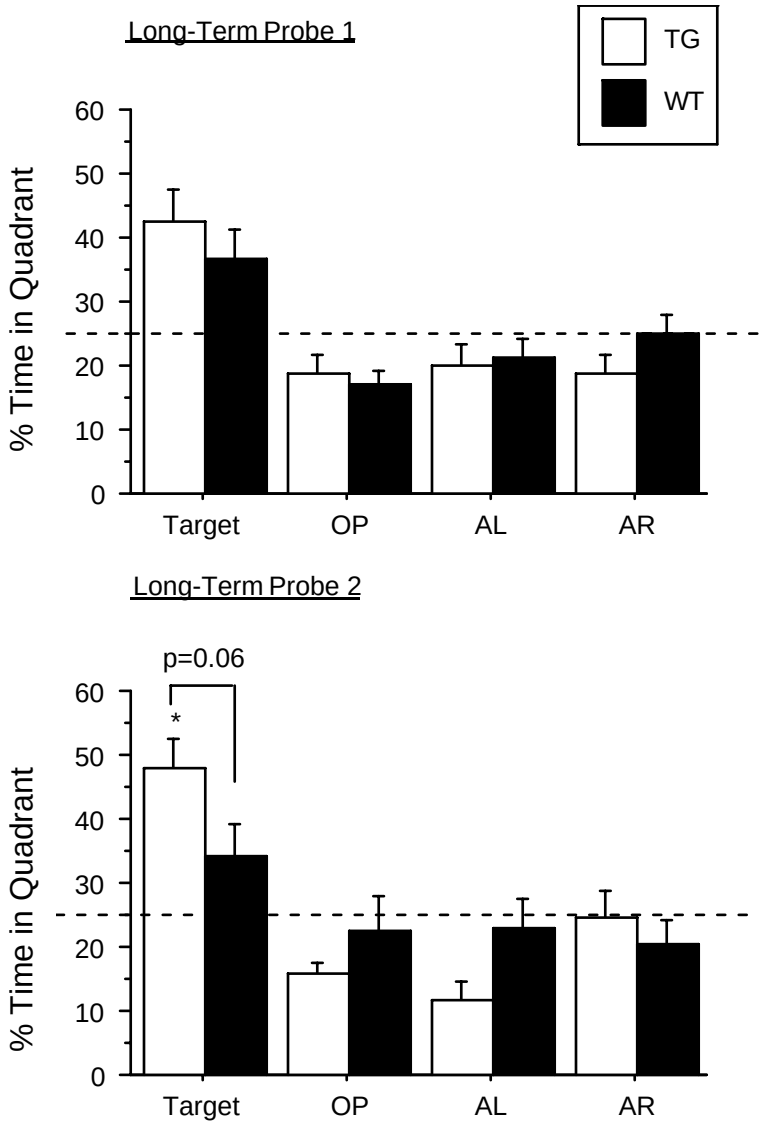



**Figure 2-14 There are no significant changes in long-term memory in MRov mice**

A 60-second probe trial was completed 2.5 weeks and 5 weeks after the last day of training. During long-term probe trial 1, both groups spent significantly more time in the quadrant where the platform used to be (Target). During long-term probe trial 2, MRov (TG) mice spent more time in the Target quadrant and WT mice did not, however there was a strong trend towards a difference between the groups ($p = 0.06$). The dashed line (25%) represents random, or "chance" performance. (OP) Opposite; (AL) adjacent left; and (AR) adjacent right quadrants. *= $p < 0.05$ relative to 25% chance performance.

**Discussion:**

There are no significant differences between MRov and WT mice in the hidden platform version of the MWM. It should be noted, however, that MRov mice did exhibit a trend towards increased long-term memory at the 5 week probe trial as compared to the WT mice. These results are similar to another model of forebrain specific overexpressionof MR, where a mild, but significant, cognitive improvement was observed with transgenic (MT-tg) mice spending more time in the target quadrant relative to WT mice during the probe trial 24 hours after the final day of training (Lai, Horsburgh et al. 2007). There are, however, two main differences between our MRov mice and MR-tg mice that may account for the differences in results. First, MRov mice display a roughly 20-25% increase in MR mRNA expression in the hippocampus relative to WT littermates. In contrast, MR-tg mice were reported to exhibit a 474% increase in MR mRNA expression averaged over regions CA1-CA3 relative to its WT littermate controls. While both sets of transgenic mice displayed decreased anxiety-related behaviors, the lack of a change in learning and memory in MRov mice may be due to a dose dependent effect, with the level of overexpression of MR in the hippocampus not reaching a critical threshold to observe a similar cognitive improvement as MR-tg mice. Second, MRov mice are different from MR-tg mice in that GR mRNA in region CA1 of the hippocampus is significantly decreased relative to WT mice in MRov mice, but there is no difference in hippocampal GR between MR-tg mice and their respective WT controls. GR activity is also known to contribute to processes underlying learning and memory and it may be this long-term decrease in GR in CA1 of MRov mice, combined with the

increase in MR, may represent a compensatory mechanism that leads to relatively normal learning and memory processes in the MWM in MRov mice.

Berger and colleagues found that mice with a forebrain specific deletion of MR exhibited decreased performance during the acquisition phase of the MWM, but no differences in the recall phase during the probe trials (Berger, Wolfer et al. 2006). Given that the loss of MR in these animals is complete in the hippocampus, as well as other brain regions such as the amygdala, the forebrain-specific MR-KO mice represent a model with a more drastic change in glucocorticoid signaling than the MRov mice. Thus, the altered learning and memory in forebrain MR-KO but not in MRov mice may also be due to dose dependent effects.

The analysis of learning and memory in this experiment was in young animals (4-5 months) under baseline conditions. Under conditions of sustained increase in corticosteroids, such as chronic stress, there are significant impairments in learning and memory (Sousa, Lukoyanov et al. 2000), and studies have shown that MR levels are decreased under conditions of chronic stress (Lopez, Chalmers et al. 1998). While the level of MR overexpression may be too low to affect learning and memory in young MRov mice under baseline conditions, it remains possible that the sustained increase in MR levels in MRov mice may prevent or attenuate the cognitive deficits that accompany chronic stress. Similarly, aging has been shown to impair learning and memory and the effects of sustained increases in MR may be more readily assessed in aged MRov and WT mice. Finally, MR activity has also been shown to be involved in processes underlying contextual fear-conditioning, a different type of hippocampal dependent task, and it may be possible that MR overexpression in MRov mice could affect aspects of

learning and memory in this context. These hypotheses will be further tested in future studies.

# Chapter 3

## Role of mineralocorticoid receptor in protection from glucocorticoid endangerment of HT-22 hippocampal cells

### *Introduction:*

Hippocampal neurons are extremely sensitive to excitotoxic and oxidative insults such as hypoxia/ischemia, seizure, and hypoglycemia. Glucocorticoid hormones released during stress have been shown to exacerbate the toxic effects of these types of neurological insults (Lee, Ogle et al. 2002). This concept of "glucocorticoid endangerment" is particularly relevant to the hippocampus, a major site of corticosteroid receptor expression. Chronic elevations in plasma cortisol are observed in multiple affective disorders including major depression, and it is has been proposed that this increase in glucocorticoids may contribute to the decreased structural and functional integrity of the hippocampus that accompanies these diseases (Lee, Ogle et al. 2002; Csernansky, Dong et al. 2006). Indeed, recent data have shown that the subtypes of depression with the highest rates of hypercortisolism are most often associated with atrophy of the hippocampus (Sheline, Sanghavi et al. 1999). However, the mechanisms by which glucocorticoids alter the vulnerability of hippocampal neurons to neurological insults remains poorly understood.

The actions of glucocorticoids are mediated by two types of receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). MR and GR are members of the steroid/thyroid hormone receptor superfamily and they function as ligand-activated transcription factors. Both MR and GR bind the rodent glucocorticoid hormone corticosterone (cortisol in humans), but they do so with very different affinities (0.5nM for MR and 5nM for GR), leading to different activation profiles throughout the circadian rhythm and during times of stress. These varied activation patterns often result in differential cellular responses to varying corticosteroid concentrations. As MR and GR are co-expressed in numerous hippocampal subfields, understanding their independent and overlapping roles in hippocampal function is critical.

Recent observations suggest that differential activation of MR and GR can have profoundly different effects on neuronal survival. While high dose glucocorticoid treatment or stress increases vulnerability to neuronal damage, there is substantial evidence that this glucocorticoid endangerment is mediated by GR, but not MR (Herman and Seroogy 2006). For example, *in vitro* studies indicate that glucocorticoid enhancement of neurotoxicity is inhibited by the GR antagonist RU486 but not by MR antagonists (Talmi, Carlier et al. 1995). In addition, administration of GR (but not MR) agonists is sufficient to enhance neurotoxic damage in the hippocampus (Goodman, Bruce et al. 1996).

In contrast, a number of studies suggest that the low levels of corticosteroid that activate MR may play a trophic or protective role (Woolley, Gould et al. 1991). Overexpression of MR in the hippocampus of transgenic mice provides protection from ischemic insult relative to wildtype controls (Lai, Horsburgh et al. 2007). Similarly,

overexpression of MR in the neuronal cell line PC12 provides protection from various neurological insults (Lai, Horsburgh et al. 2007). Interestingly, in both rats that have received chronic unpredictable stress and in suicide victims with a history of depression, the expression levels of MR have been shown to be decreased, leading overall to an imbalance in the MR/GR ratio (Lopez, Chalmers et al. 1998). These data support the hypothesis that a balance in the activation of MR and GR in forebrain regions such as the hippocampus is important in the maintenance of proper physiological and cellular homeostasis (De Kloet, Vreugdenhil et al. 1998), including neuronal survival.

Neurological insults such as ischemia and hypoglycemia are thought to be injurious to cells through a mechanism that involves an over-accumulation of the excitatory amino acid glutamate. Glutamate has been shown to be toxic to neuronal cells via two different mechanisms. In the first mechanism, excessive binding of glutamate to specific glutamate receptors produces excitotoxic effects through a rise in cytosolic calcium and the production of oxygen radicals (Lipton 1999). Alternatively, glutamate toxicity can also occur when excessive levels of the amino acid compete with cystine at the cystine/glutamate transporter, resulting in an antioxidant imbalance in the cell (Murphy, Miyamoto et al. 1989). Decreased cystine transport results in the decreased production of glutathione, leading to an increase in reactive oxygen species and increased calcium influx, ultimately leading to cell death through both apoptotic and necrotic mechanisms (Tan, Wood et al. 1998).

The immortalized mouse hippocampal cell line HT-22 has been shown to be sensitive to glutamate-induced oxidative stress via competition at the cystine/glutamate transporter. Activation of GR in the HT-22 hippocampal cell line exacerbates

glutatmate- induced cell death (Behl, Lezoualc'h et al. 1997), and this effect is blocked by a GR antagonist. However, the role of MR in the corticosteroid-mediated increase in glutamate-induced cell death has not been previously examined. In this study, we demonstrate that HT-22 cells express GR, but not MR. To study the potential neuroprotective actions of MR in corticosteroid-mediated effects on glutamate toxicity, we therefore stably transfected HT-22 cells with a CMV-MR construct to generate clones that co-express MR and GR at varying levels. These clonal cell lines were characterized for MR/GR levels, transactivation properties, and their sensitivity to corticosteroid-mediated exacerbation of glutamate-induced cell death. Additionally, the potential role of apoptotic mechanisms in the glucocorticoid-mediated effects was also examined.

***Materials and Methods:***

**Cell culture.** HT-22 cells were obtained from Dr. David Schubert (Salk Institute, San Diego). This immortalized mouse hippocampal cell line was selected from HT-4 cells based on glutamate sensitivity (Maher and Davis 1996). HT-22 cells were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS, Hyclone, Logan, UT), and gentamicin (50μg/ml, Invitrogen) at 37°C in 5% $CO_2$.

**Generation of HT22/MR Stable Expression Clones.** The cloning of the full-length MR cDNA (MR2A) was previously described (Rozeboom, Akil et al., 2007). MR2A was subcloned into a CMVneo expression construct (kindly provided by Mike Uhler) to generate CMV-MR. To generate clones of HT-22 that stably express MR, cells were cotransfected with CMV-MR and a plasmid that expresses the puromycin resistance gene

using Lipofectamine (Invitrogen) according to the manufacturer's protocol. Twenty-four hours later, media was replaced with media containing 2.5 μg/ml puromycin. This media was replaced every 3-4 days for 10 days. Individual clones were isolated and grown individually for another 10-14 days in puromycin-containing media.

**Transfections.** HT-22 Parent, HT-22/MR24 and HT-22/MR16 cells were plated at 1.5-2.5Z$10^4$ cells/well in 24 well plates in media containing 10% FCS. Twenty-four hours later, transient transfections were performed using Lipofectamine. Briefly, 250 μg of a glucocorticoid responsive reporter plasmid (5GRE-Luc or MMTV-Luc) and 50ng of CMV-β-gal were transfected per well in serum free media. After 12 hours, the transfection media was replaced with media containing 10% charcoal-stripped FCS and the drugs indicated (or vehicle alone) for an additional 20 hours. Forty-eight hours after transfection, cells were rinsed with ice cold PBS-D and lysed in the well with buffer containing 0.25M Tris pH 8.0, 0.1 mM EDTA, 5mM $MgSO_4$, 1mM DTT, and 1% Triton. Lysates were centrifuged at 10,000 rpm for 10min at 4°C and supernatants were assayed for luciferase activity as previously described (Rozeboom, Akil et al., 2007) using a Promega 20/20n luminometer. RLU values were normalized to β-gal activity to correct for transfection efficiency as previously described (Seasholtz, Thompson et al. 1988). Data for all treatment groups are expressed as fold induction over vehicle alone. Experiments were performed in triplicate and repeated 2-5 times.

**Corticosterone Binding assays.** For single receptor binding capacity determinations, 3X$10^5$ cells were plated in 10% charcoal-stripped FCS. Twenty-four hours later, cells

were harvested and resuspended in TEMDG (20 mM Tris/1 mM EDTA/20 mM sodium molybdate/5 mM DTT/10% glycerol/10x Sigma Protease Inhibitor Cocktail, pH 7.6 at 4°C) binding buffer. After a brief sonication (setting 4 for 5 seconds with a Branson Sonifier 150), samples were centrifuged at 4 C at 16,100 x g for 30 min (Pearce and Yamamoto 1993). Supernatants were removed and used for the binding assays. For each sample, three sets of lysates were incubated, in triplicate, for 20 hours at 4°C under the following 3 conditions: 1) 20nM [1,2,6,7-$^3$H] corticosterone (67μCi/nmol, Amersham, Arlington Heights IL) (total binding), 2) 20nM [$^3$H]corticosterone + plus 100 nM mifepristone to displace GR binding, and 3) 20nM [$^3$H]corticosterone + 10μM unlabeled corticosterone for determination of nonspecific binding. Bound and free [$^3$H]corticosterone were separated by addition of an ice-cold dextran/charcoal suspension in TEMDG (0.05/0.5%). The tubes were incubated 10 min at 0-2°C and centrifuged. The supernatants were removed and counted in a Packard scintillation counter at 45% efficiency. Protein content was measured by Bradford assay using BSA as a standard, and the apparent maximal binding capacity ($B_{max}$) was expressed as fmol/mg protein. GR binding (RU486 displaceable) was calculated from the difference between conditions 1 and 2 above. MR binding was calculated from the difference between condition 2 ($^3$H-corticosterone binding not displaced with RU486) minus background (condition 3).

**Cell Survival Assay.** The MTT (3-(4,5,dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay (Hansen, Nielsen et al. 1989) was used to measure cell death. This assay relies on the conversion of the MTT tetrazolium salt to a purple formazan crystal by mitochondrial enzymes. HT-22 Parent and HT-22/MR16 cells were plated at 8-15X10$^3$

cells/well in 24 well plates in low glucose DMEM + 10% charcoal-stripped FCS media. The next day, cells were pre-treated with the drugs indicated or vehicle alone for 24 hours. Cells were then treated with varying concentrations of glutamate for an additional 16-22 hours. MTT (Sigma, 5mg/ml stock in PBS) was added to 0.05mg/ml final concentration for 4 hours. Finally, 500µl of solubilization buffer (50% dimethylformamide and 20% SDS, pH 4.8) was added. After overnight incubation at 37°C, absorbance readings were measured at 590nm. Data are expressed as % survival relative to vehicle treated control cells. All experiments were performed in triplicate and repeated multiple times.

**Western Blots.** HT-22 Parent and HT-22/MR16 cells were plated on 10 cm plates at a density of $22.5X10^4$ cells/plate. Thirty-six hours later, cells were pre-treated with 1µM corticosterone or ethanol vehicle alone for 24 hours. Six mM glutamate was added (or vehicle control) and cells were harvested at 2-hour intervals for 12 hours. Cells were harvested in 1ml cold PBS, centrifuged at 4°C at 3000 rpm for 8 minutes, and cell pellets were re-suspended in 100 µl RIPA buffer (0.05M Tris pH 7.5, 0.15M NaCl, 1% IGEPAL, 0.1% SDS, 1% deoxycholic acid) plus 1:50 dilution of protease inhibitor cocktail (Sigma). Lysates were centrifuged at 10,000 rpm for 10 minutes at 4°C and supernatants were used for western analysis. Equal amounts of protein (12 µg) were loaded and separated on discontinuous 10% (bottom 1/3), 7% (top 2/3) SDS-polyacrylamide gels. Proteins were tank transferred to PVDF membranes (Millipore, Billerica MA) in transfer buffer (25mM Tris, 150mM glycine, and 20% methanol) + 0.02%SDS for 2 hours at 200V. For immunoblots, membranes were blocked in a buffer

containing 20mM Tris pH 7.5, 150mM NaCl, 0.1% Tween 20 (TBST) + 5% non-fat dry milk (NFDM) for 1hr at room temperature and then incubated with a 1:2000 dilution of α-Fodrin antibody (Biomol-Affinity Research Products, Plymouth Meeting, PA) overnight at 4°C in TBST. After washing, immunoblots were incubated with a 1:50,000 dilution of HRP linked donkey anti-mouse antibody (Jackson, West Grove, PA) for 1-2 hours at room temperature in TBST. Immunoblots were visualized with Lumi-Light (Roche, Indianapolis, IN) according to the manufacturer's suggestions.

***Results:***

**Generation of HT-22 MR expressing clones:**

The HT-22 hippocampal cell line has previously been shown to exhibit corticosterone-mediated transactivation of MMTV-luciferase activity that is blocked by RU486 (Schmidt, Holsboer et al. 2001), consistent with the presence of GR in these cells as shown by western blot (Wang, Pongrac et al. 2002). However, previous studies had not tested for the presence of MR in HT-22 cells. Therefore, [$^3$H]-corticosterone binding assays were performed with the HT-22 Parent cell line. RU486 blocked > 99% of [$^3$H]-corticosterone binding, demonstrating a lack of detectable corticosterone binding by MR in the HT-22 cells (Figure 3-1A). Therefore, to address the effects of corticosteroid signaling in a cellular context that contains both MR and GR, more closely resembling the endogenous hippocampal setting, HT-22 cells were stably transfected with a CMV-MR expression construct. [$^3$H]-corticosterone binding assays were used to characterize the apparent maximal binding ($B_{max}$) of MR and GR in the independently selected clonal

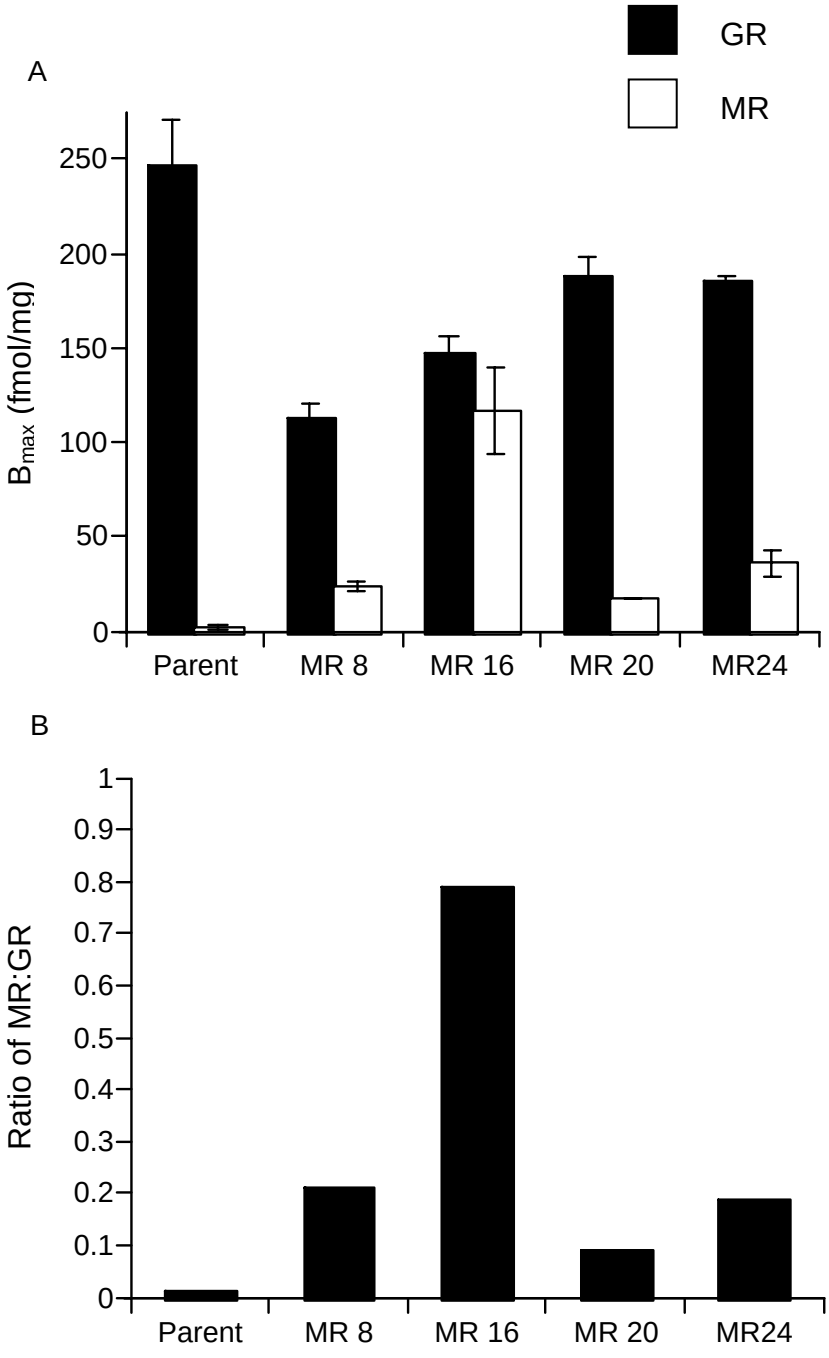



**Figure 3-1 HT-22 Parent and HT-22/MR clones express either GR alone or MR and GR in different ratios.**

A) Lysates from HT-22 Parent or HT-22/MR clones were incubated with [$^3$H]corticosterone plus or minus RU486 to specifically block GR binding, or unlabeled corticosterone to determine non-specific binding as described in the Materials and Methods. B) Binding data from (A) expressed as ratios of MR:GR. GR binding was derived from the RU486 displacement of the [$^3$H]corticosterone binding and MR binding was determined from the difference between total and GR [$^3$H]corticosterone binding.

cell lines. While the $B_{max}$ for GR in HT-22 Parent cells was approximately 250fmol/mg protein, there was no detectable binding for MR (Figure 3-1A). Analyses of four stably transfected clonal cell lines reveals a range of $B_{max}$ values for MR binding, with $B_{max}$ values ranging from 10-110 fmol/mg protein. In all of these clones, $B_{max}$ values for GR are decreased relative to the parent cell line, consistent with the MR mediated down-regulation of GR (Herman and Spencer 1998). Figure 3-1B depicts the binding data as a ratio of [$^3$H]-corticosterone binding by MR relative to GR, revealing ratios that range from 0 in the parent cell line to an MR binding at 80% that of GR in clone HT-22/MR16.

**Transactivation properties of HT-22 cells and HT-22/MR stable clones:**

To further characterize the HT-22 Parent and clones, reporter gene transactivation assays were performed with two different promoters driving the expression of luciferase. The 5GRE-Luc construct contains 5 tandem repeats of the tyrosine amino transferase GRE in combination with the TATA region of the Adenovirus 2 major late promoter and the MMTV-Luc construct contains the well characterized glucocorticoid responsive MMTV-LTR promoter. Using a wide range of corticosterone doses, inductions of reporter gene activity with the 5GRE promoter were not detectable in the HT-22 parent cells until 100nM corticosterone (Figure 3-2A), similar to what has been previously reported (Schmidt, Holsboer et al. 2001); in clone HT-22/MR16, inductions in GRE-luciferase reporter gene activity were detected as low as 0.01 nM corticosterone. The HT-22/MR24 clone showed intermediate levels of induction over the dose response curve, relative to HT-22/Parent and HT-22/MR16, with statistically significant inductions at 0.05nM corticosterone. Results using the MMTV-luciferase construct were similar to

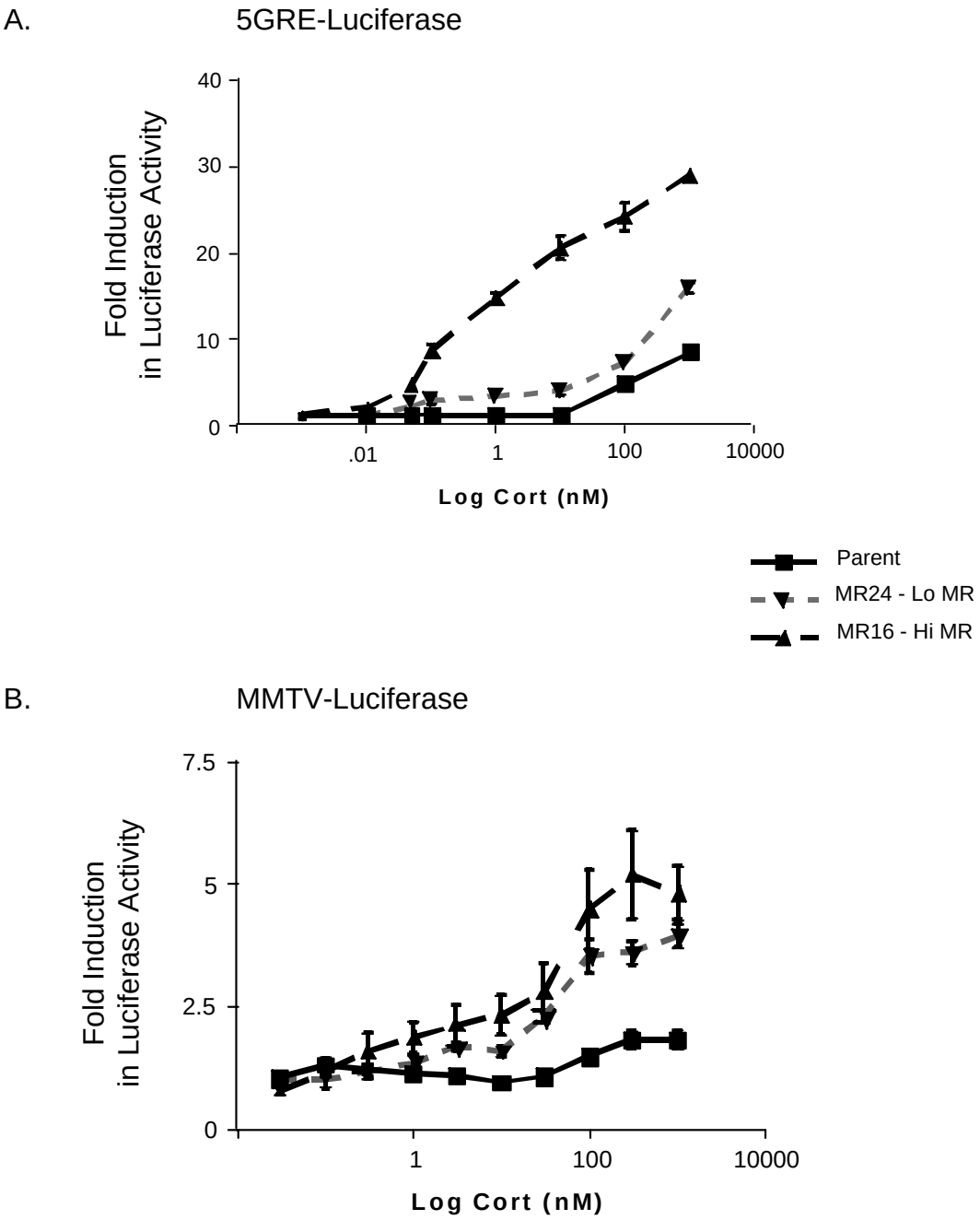



**Figure 3-2 HT-22 Parent, HT-22/MR16, and HT-22/MR24 clones exhibit different transactivation profiles in response to different doses of corticosterone.**

(A) The different cell lines were transiently transfected with the 5GRE-Luc and CMV β-gal for 12 hours and exposed to varying concentrations of corticosterone for an additional 20 hours. Normalized luciferase activity (luciferase activity/ gal activity) for samples was divided by their respective no drug control to result in fold induction in luciferase activity. The data are presented as mean ± SEM and represent three independent experiments done in duplicate. B) Experiment performed identically to (A) except with the MMTV-Luc construct.

the 5GRE-luciferase construct (Figure 3-2B). Inductions in reporter gene activity did not rise above background until 100nM corticosterone in the HT-22 parent cells; significant inductions in luciferase activity were observed at less than or equal to 1nM corticosterone in both HT-22/MR16 and HT-22/MR24 (Figure 3-2B). As seen above, clone HT-22/MR24 which contains about 3-4 fold less MR than clone HT-22/MR16, exhibited an increase in reporter gene activity at similarly low levels of corticosterone as MR16, albeit the fold induction in luciferase activity was less. As the Kd of MR for corticosterone is 0.5nM, luciferase activity at this concentration of drug most likely reflects the activation of MR, consistent with the lack of induction in reporter gene activity until much higher concentrations in the parent HT22 cell line. The differences in profiles of GRE-luc and MMTV-luc most likely reflects other contextual differences of the promoters in terms of both the number and positioning of GREs, as well as the possible presence of other cis-acting elements that may result in differential interactions of MR and GR with other co-regulators and transcription factors.

To demonstrate the receptor specificity of these transactivation properties, the GR and MR specific ligands, dexamethasone and aldosterone, and the GR-specific and MR-specific antagonists, RU486 and eplerenone were also used. In the HT-22 parent cell line, 7 and 15 fold inductions of luciferase activity from the 5GRE-luc-reporter plasmid were seen with 1μM corticosterone and dexamethasone, respectively. These inductions were blocked 100% by the co-incubation of 1μM RU486 (Figure 3-3A), but are not blocked at all by the co-incubation of the MR-specific antagonist eplerenone (data not shown). Aldosterone (10 nM) had no effect on the HT22 parent clone (Figure 3-3A). In clone HT-22/MR24 and HT-22/MR16, however, aldosterone significantly increased

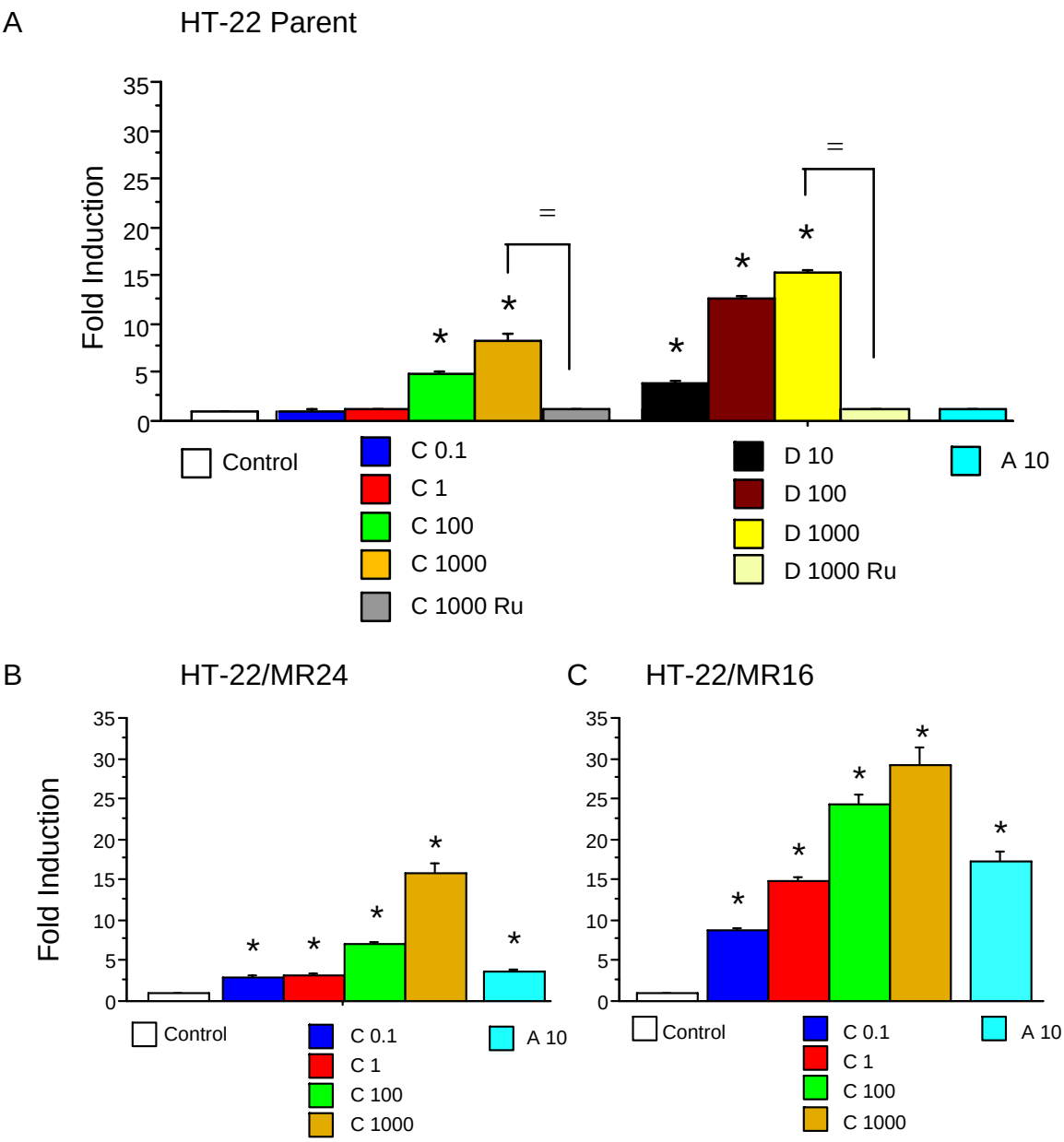



**Figure 3-3 Receptor specificity of corticosteroid transactivation properties.**

A) Parent HT-22 cells are transiently transfected with 5GRE-Luc and CMV β-gal as in Figure 2, and treated with vehicle alone, dexamethasone (D), corticosterone (C), or aldosterone (A) plus or minus RU486 as indicated. The data represent the mean ± SEM of three experiments performed in duplicate. B) Clone HT22/MR24 and C) HT22/MR16 treated with corticosterone or aldosterone as indicated. The data represent the mean ± SEM of three experiments performed in duplicate. * = $p < 0.05$ from vehicle treated cells, = = $p < 0.05$ relative to indicated dose.

luciferase activity (Figure 3-3B and Figure 3-3C). Interestingly, eplerenone effectively blocks 100% of the corticosterone-mediated inductions in luciferase activity in HT-22/MR16 at 1nM corticosterone, but neither eplerenone alone, nor RU486 alone, blocks the inductions in luciferase activity at 1 μM (data not shown). When RU486 and eplerenone were used together, they produced a significant 35% drop in reporter gene activity but this induction was still significantly above control (data not shown). These data suggest that at concentrations of corticosterone sufficient to activate both receptors, the transactivation properties of MR and GR on this promoter are more complex than with activation of either MR or GR alone. As heterodimers are likely to be forming at the high corticosterone concentrations, these results also suggest that the antagonists may not effectively block transactivation of GR/MR heterodimers.

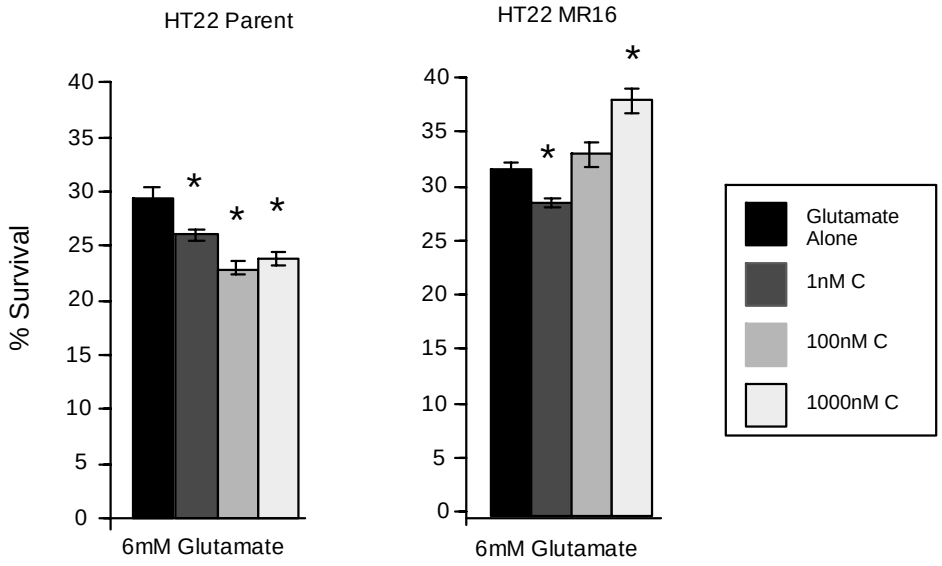
**Glutamate-induced neurotoxicity and corticosteroid endangerment and protection:**

HT-22 cells are sensitive to glutamate through a non-excitotoxic pathway, as these cells do not express ionotropic glutamate receptors. Instead, excess glutamate is thought to compete for cystine, preventing its import into the cell, thus leading to a reduction in glutathione levels, the accumulation of reactive oxygen species and eventual cell death through an apoptotic pathway. Previous studies in HT22 cells have shown that pre-exposure with corticosterone can exacerbate glutamate toxicity (Behl, Lezoualc'h et al. 1997). HT-22 cells were pre-exposed for 24 hours to different doses of corticosterone before the addition of 6mM glutamate for an additional 16 hours. As predicted, corticosterone treatment of HT-22 cells decreases, in a dose dependent manner, cell survival relative to glutamate treatment alone as measured by the MTT assay

(Figure 3-4A). To address the consequences of the activation of MR along with GR, HT-22/MR16 cells were also pre-exposed to the same doses of corticosterone before the addition of 6mM glutamate. In contrast to HT-22 Parent cells, corticosterone dose dependently increased cell survival relative to glutamate treatment alone in HT-22/MR16 cells (Figure 3-4A). To determine if corticosterone exacerbation of glutamate toxicity is dependent on the dose of glutamate, HT-22 Parent and HT-22/MR16 cells were pre-exposed with 1μM corticosterone for 24 hours before the addition of increasing levels of glutamate for an additional 22 hours. While corticosterone pre-exposure further decreased cell survival in the HT-22 Parent cell line at all 3 doses of glutamate (Figure 3-4B), corticosterone did not exacerbate cell death relative to glutamate alone in clone HT22/MR16 (Figure 3-4B), suggesting a potential neuroprotective role for MR.

Previous data suggest the involvement of apoptotic mechanisms in glutamate-induced cell death of HT-22 cells, as inhibitors of the apoptotic enzyme calpain can block glutamate-induced cell death in this cell line (Zhang and Bhavnani 2006). Preliminary experiments have been performed to address the possible role of apoptotic mechanisms in the corticosterone-mediated exacerbation of glutamate-induced cell death. HT-22 Parent and HT-22/MR16 cells were analyzed by western blots for the calpain and caspase-3 dependent cleavage of the cytoskeletal protein α-Fodrin. Cleavage of the 240KDa α-Fodrin by calpain and caspase-3 results in 145KDa and 120KDa products, respectively. HT-22 Parent cells were pre-incubated with corticosterone or ethanol vehicle alone for 24 hours before the addition of 6mM glutamate. Western blot analysis for α-Fodrin in cells harvested in two-hour intervals for 12 hours after addition of glutamate suggests that pre-incubation with corticosterone may enhance the accumulation of cleavage products

A




B

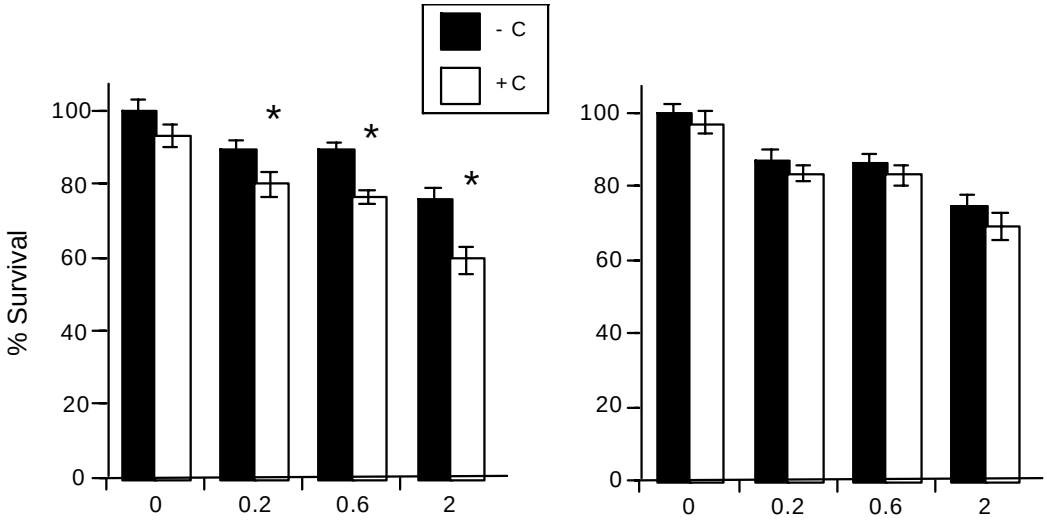



**Figure 3-4 Glucocorticoid exacerbation of glutamate toxicity is attenuated in Clone HT-22/MR16.**

A) HT-22 Parent and Clone HT-22/MR16 are pre-incubated for 24 hours with varying doses of corticosterone or vehicle alone before the addition of 6 mM glutamate for and additional 16 hours as described in the Materials and Methods. B) HT-22 Parent and Clone HT-22/MR16 cells are pre-incubated with 1μM corticosterone or vehicle alone for 24 hours before the addition of different doses of glutamate for an additional 22 hours. Data represent the mean ± SEM of 2-4 experiments all performed in triplicate.

specific to both caspase-3 and calpain activation (Figure 3-5). Similar analysis with HT-22/MR16 cells suggests no differences in the accumulation of cleavage products between cells that were pre-exposed to corticosterone and those that were exposed to vehicle.

***Discussion:***

While there is some evidence that corticosteroids can be directly toxic to neurons, the primary actions of glucocorticoids are thought to place neurons in a state of metabolic distress that subsequently exacerbates any further insults, such as enhancement of glutamate toxicity. Glutamate levels are increased during insults such as ischemia and hypoglycemia and excessive levels of corticosteroids during such insults increases neuronal cell death in hippocampus (Sapolsky and Pulsinelli 1985). The HT-22 mouse hippocampal cell line is quite sensitive to glutamate toxicity, and it has been shown that corticosterone pre-exposure of these cells exacerbates glutamate toxicity. Hence, these cells provide a model system in which to examine the role of corticosteroid receptors in corticosterone-induced endangerment to glutamate-induced cell death. However, the parent HT-22 cells express only GR, suggesting that glucocorticoid endangerment is mediated entirely by GR in this cell line. As GR is co-expressed with MR in most regions of the hippocampus, the parent HT-22 cells do not recapitulate the normal corticosteroid-signaling environment of the hippocampus. The goal of our study was to test the hypothesis that expression and activation of MR in HT-22 cells may attenuate or block the endangering effects of GR signaling alone.

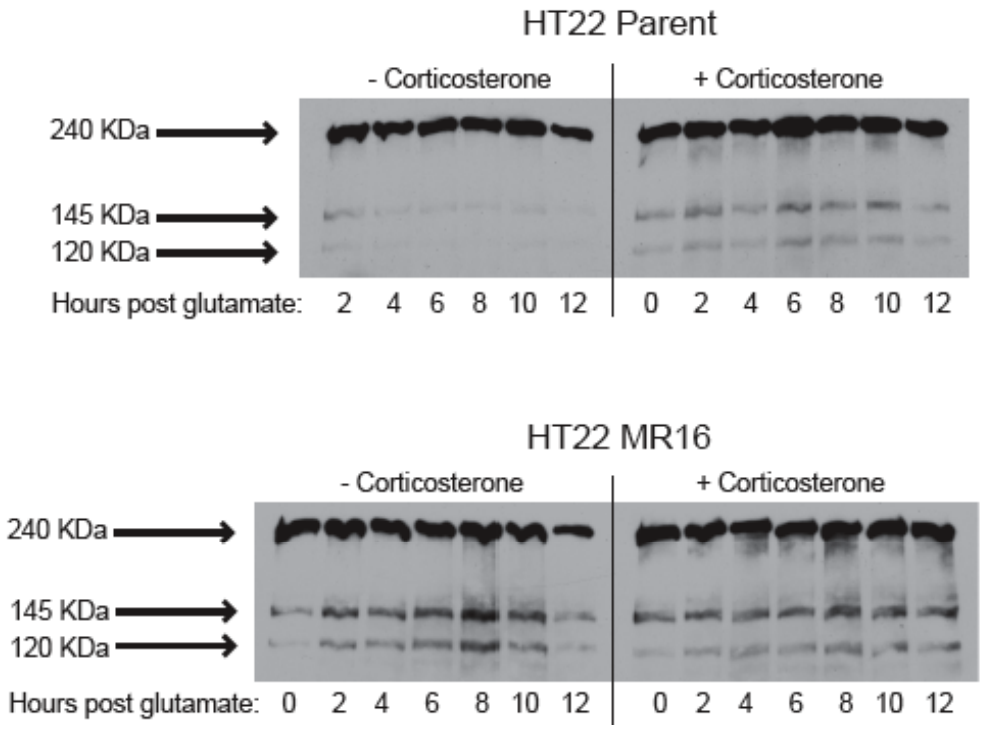



**Figure 3-5 Corticosterone pre-treatment enhances α-Fodrin cleavage in HT-22 Parent but not HT-22/MR16 cells.**

HT-22 Parent and HT-22/MR16 cells were pre-treated with either 1μM corticosterone or vehicle alone (- corticosterone) for 24 hours before the addition of 6mM glutamate. Cells were harvested after the indicated amount of time. Cell extracts were separated by SDS-PAGE and used for western blot analysis with an anti- α-Fodrin antibody. The 240 KDa band represents the uncleaved protein, whereas the 145 KDa and 120 KDa bands represent α-Fodrin cleaved by activated calpain and caspase-3, respectively.

We report here the generation of stably-transfected HT-22 clones with varying levels of MR, and hence different MR/GR ratios, as evidenced by [$^3$H]corticosterone binding. Previous transactivation studies have shown that, depending on the cellular and promoter context, MR activation can have synergistic effects with GR when both are activated (Trapp, Rupprecht et al. 1994), or it can have antagonistic effects, repressing the transactivation activity of GR alone (Liu, Wang et al. 1995). In the context of HT-22 cells, as the ratio of MR to GR increased from 0 in the Parent cell line to 0.2 and 0.8 in clones HT-22/MR24 and HT-22/MR16 respectively, the level of 5GRE-luciferase and MMTV-luciferase reporter gene activity is increased over the entire corticosterone dose response curve. These data suggest that at low concentrations of corticosterone, MR is capable of mediating positive transcriptional effects on these two promoters; similarly at high levels of corticosterone, sufficient to bind both MR and GR, the activity of MR does not inhibit GR function from the two promoters analyzed, but significantly increases the transcriptional response.

HT-22 cells are very sensitive to glutamate and Behl et al. showed that pre-incubating HT-22 cells with corticosterone leads to an exacerbation of the glutamate toxicity that is dependent on GR activation alone (Behl, Lezoualc'h et al. 1997). Here we confirm that finding, showing that corticosterone enhances glutamate-induced cell death in the HT-22 Parent cell line. However, in HT-22/MR16, pre-incubating the cells with 1μM corticosterone no longer enhances glutamate-induced toxicity at any glutamate concentration tested, suggesting that the activation of MR along with GR is capable of diminishing the glucocorticoid endangerment effect.

Extensive efforts have been made to determine the molecular mechanisms underlying glutamate mediated cell death in HT22 cells. These mechanisms are complex and are thought to involve the initial depletion of GST, followed by activation of 12-lipoxygenase, and subsequent accumulation of ROS and intracellular $Ca^{2+}$ (Li, Maher et al. 1997). Ultimately, morphological features of both apoptosis and necrosis have been described in the cell death pathway (Tan, Wood et al. 1998). In addition, molecular features of apoptosis have also been described in the glutatmate-induced cell death pathway. This is exemplified by DNA fragmentation as well as involvement of apoptotic proteases, as inhibitors of calpain can prevent glutamate-mediated features of apoptosis in HT-22s (Zhang and Bhavnani 2006). The mechanisms by which glucocorticoids exacerbate glutamate toxicity in HT-22s are not fully understood, but evidence suggests that metabolic constraints may be involved as increasing the concentration of glucose in the media can attenuate the glucocorticoid endangerment effect (Behl, Lezoualc'h et al. 1997). Preliminary studies suggest that pre-incubation of HT-22 Parent cells with corticosterone may increase cell death in response to glutamate via apoptotic mechanisms, as the presence of α-Fodrin cleavage products is increased throughout the time-course of glutamate treatment relative to cells not treated with corticosterone. Interestingly, the accumulation of α-Fodrin cleavage products in HT-22/MR16 cells is not increased in cells pre-treated with corticosterone relative to vehicle treated cells. MR activation has been previously implicated in neuronal protection from toxic insults (Lai, Seckl et al. 2005; Lai, Horsburgh et al. 2007). MR activity has also been implicated in the regulation of select cell survival and cell death related genes in the hippocampus (McCullers and Herman 1998). These data suggest that the activation of MR along with

GR may normalize processes that would otherwise lead to increased apoptosis if only GR were activated during a toxic insult. Indeed, using serial analysis of gene expression (SAGE) in the rat hippocampus, a previous study found that when MR and GR are activated at the same time, different but partially overlapping sets of genes were regulated by corticosterone relative to when MR was activated alone (Datson, van der Perk et al. 2001).

Currently, evidence suggests that prolonged exposure to high levels of corticosteroids may be involved in the decline of the structural and functional integrity of the hippocampus and that these alterations may be an important component in the etiology of mood disorders. It is hypothesized that subtle alterations in the ratio of MR to GR expression and activation in the hippocampus may underlie some of these deleterious effects. Experiments with HT-22 cells have shown them to be a good model system to study the endangering effects of corticosteroids and their ability to exacerbate subsequent toxic insults. Evidence provided here suggests that when MR is stably expressed in HT-22 cells, the ability of corticosteroids to exacerbate glutamate toxicity is attenuated. These data support the hypothesis that a proper balance in the activation of MR and GR in hippocampal cells has important cellular consequences and that the declines in the ratio of MR to GR observed in models of depression and anxiety, such as chronic unpredictable stress, may serve to enhance the phenomenon of glucocorticoid endangerment.

## Chapter 4

## Comparison of corticosteroid-responsive genes in mouse hippocampal cell lines that either express the glucocorticoid receptor alone or both the glucocorticoid and the mineralocorticoid receptor

### *Introduction:*

Glucocorticoid hormones influence the structure and function of the hippocampus, a brain region intimately involved in learning and memory and the modulation of anxiety-related behaviors. The high sensitivity of the hippocampus to glucocorticoids is not surprising as the hippocampus exhibits the highest levels of co-expression of the two receptors that bind glucocorticoid hormones, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). MR and GR are two of the most closely related members of the steroid/thyroid hormone receptor superfamily and they function as ligand-activated transcription factors. These two corticosteroid receptors normally reside in the cytoplasm, dimerize upon ligand binding, and translocate to the nucleus where they can enhance or repress transcription of glucocorticoid-responsive genes.

While both MR and GR bind glucocorticoid hormones, they do so with very different affinities (MR: Kd~0.5nM for corticosterone; GR: Kd~5nm for corticosterone),

suggesting that they have different activation profiles throughout the circadian rhythm and during times of stress. In addition to differences in ligand binding properties, MR and GR are also significantly different in their N-terminal domains (only 15% amino acid identity), regions that contain activating function (AF) sequences that allow for interactions with other transcription factors and co-regulators. These distinct AF regions allow similar interactions for MR and GR with some co-regulators, while other co-regulators have been found to only interact with one receptor (Kitagawa, Yanagisawa et al. 2002), or to act as a coactivator of one but a corepressor of the other (Pascual-Le Tallec, Simone et al. 2005). Hence, these differences in ligand affinity and interactions with co-regulators define mechanisms by which MR and GR can differentially affect transcription of glucocorticoid-responsive genes when expressed individually or together.

Finally, MR and GR have been shown to form heterodimers, in addition to homodimers, in contexts where they are coexpressed such as the hippocampus. Little is known about the rules that govern heterodimerization beyond stoichiometric considerations. It is known, however, that the transcriptional outcome of heterodimer formation for transcriptional activation or repression is dependent on the cellular and/or promoter contexts and corticosterone concentrations. In some cases, heterodimers work more efficiently than either GR or MR homodimers (Trapp, Rupprecht et al. 1994) and in other cases, the heterodimer is less efficient than the homodimers (Liu, Wang et al. 1995). Overall, the data suggest that when MR and GR are co-expressed, the transcriptional regulation of corticosteroid-responsive genes will be determined by multiple factors including the concentration of corticosteroid, the relative levels of MR and GR, and the expression profiles of other transcription factors and coregulators. All of

these factors together may result in the differential regulation of genes when MR and GR are both activated relative to the regulation derived from the activation of either MR or GR alone.

Functionally, a shift in the balance of MR and GR activation can have significant effects on several features of cellular function and survival. On a cellular level, glucocorticoids can modulate multiple properties relevant to learning and memory and information transfer out of the hippocampus. Levels of corticosteroids equivalent to a stressor decrease long-term potentiation (LTP) whereas low levels of corticosteroids enhance LTP (McEwen and Sapolsky 1995). Similarly, low levels of corticosterone can enhance excitability of pyramidal neurons in the hippocampus while higher levels of hormone result in decreased neuronal excitability (Joels and de Kloet 1991; Joels and de Kloet 1992). Interestingly, under conditions of ADX, when no corticosteroids are present, a similar cellular phenotype is observed as under high levels of corticosteroids. These inverted U shaped patterns can be explained, at least in part, by the relative occupation of the two corticosteroid receptors present in the hippocampus. Studies with low levels of corticosteroids, which preferentially bind to MR, as well as specific agonists for MR suggest that the activity of this receptor is responsible for increased LTP and increased neuronal excitability. Conversely, higher levels of corticosteroids, activating both MR and GR, and specific agonists for GR decrease LTP and neuronal excitability (Joels and de Kloet 1991; De Kloet, Vreugdenhil et al. 1998). With respect to cell survival, high levels of glucocorticoids can enhance the likelihood that a neuron will die with a subsequent toxic insult. Multiple lines of evidence suggest that this

"glucocorticoid endangerment" is propagated through predominant activation of GR, whereas MR activation can provide a neuroprotective effect (Herman and Seroogy 2006)

Clearly, an understanding of glucocorticoid actions on hippocampal structure and function requires an understanding of the combined actions of MR and GR. As mentioned previously, the transcriptional output of MR and GR activity may be affected not only by absolute glucocorticoid concentrations, but also by the levels and ratio of MR to GR present in the cell. The study of MR/GR heterodimer-mediated transcriptional regulation has been limited to only a few individual genes (Trapp, Rupprecht et al. 1994; Liu, Wang et al. 1995; Ou, Storring et al. 2001), and little is known at the transcriptome level about genes that are regulated exclusively by the individual receptors and/or the extent of overlap of the transcriptional effects of activated MR and GR.

A recent study from Datson et al. aimed to identify corticosteroid-responsive genes in the rat hippocampus using either low or high doses of corticosterone (to activate MR alone or MR and GR together) in adrenalectomized (ADX) rats. Using SAGE analysis, they identified 203 genes that were regulated by either low or high doses of corticosterone, but only 33 genes that were regulated by both doses of corticosterone (Datson, van der Perk et al. 2001), suggesting that the activation of MR alone or GR and MR together in the same cell may regulate both distinct and partially overlapping sets of genes. A drawback to this analysis is that it does not allow the identification of genes responsive specifically to activated GR, as it is not possible to distinguish between GR homodimer and GR/MR heterodimer activities at high corticosterone concentrations. This system also does not allow for the assessment of gene expression at a constant dose of corticosterone under conditions of altered MR and GR levels. These questions can be

more easily addressed in an *in vitro* system such as a mouse hippocampal cell line.

The mouse hippocampal cell line HT-22 was originally generated to study mechanisms of glutamate toxicity. It was subsequently demonstrated that this cell line expresses GR, and it has been used to study GR-mediated glucocorticoid endangerment as well as GR interactions with other signaling pathways. We previously determined that HT-22 cells do not express MR. To address the global transcriptional effects of MR and GR signaling, we generated clones of HT-22 cells that co-express MR and GR in varying levels for use in a microarray analysis. By examining the gene expression profile of the HT-22/Parent and HT-22/MR clonal cells with low and high doses of corticosterone, this system can overcome drawbacks of the endogenous system, allowing us to identify genes responsive to GR activation (HT-22/Parent at high dose corticosterone), genes responsive to MR activation (HT-22/MR clones at low dose corticosterone) and genes regulated by the activation of both MR and GR (HT-22/MR clones at high dose corticosterone).

***Materials and Methods:***

**Cell culture.** HT-22 cells were obtained from Dr. David Schubert (Salk Institute, San Diego). This immortalized mouse hippocampal cell line was selected from HT-4 cells based on glutamate sensitivity (Maher and Davis 1996). HT-22 cells were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS, Hyclone, Logan, UT), and gentamicin (50μg/ml, Invitrogen) at 37°C in 5% $CO_2$.

**Generation of HT-22 clones containing MR.** The generation and characterization of clone HT-22/MR16 is described in detail in Chapter 3. HT-22 Parent cells express GR

but do not express MR. HT-22/MR16 clonal cells were shown by [$^3$H]corticosterone binding assays to express GR and MR at a ratio of 1:0.8.

**Isolation of RNA.** Cultures of HT-22 Parent and HT-22/MR16 cells were plated on 10 cm plates in DMEM + 10% charcoal-stripped FCS + 50 μg/ml gentamicin and grown to ~60-70% confluency. Triplicate plates of cells were treated with vehicle (ethanol, 0.1% final concentration) or corticosterone (1nM or 100nM, Sigma, St. Louis, MO) 24 h before harvest, and total cellular RNA was isolated using Trizol (Invitrogen) according to the manufacturer's protocol. Thirty μg of total RNA was further purified using the RNeasy spin columns according to the manufacturer's protocol (Qiagen, Inc., Valencia, CA).

**RNA Probes for Microarray.** Total RNA (175 ng) was amplified using the Illumina Totalprep RNA Amplification Kit (Ambion, Inc.) to generate biotinylated amplified RNA (aRNA). Two micrograms of biotinylated aRNA was used for hybridization to Illumina Sentrix Bead Chips (Illumina Mouse 6_V1) according to manufacturer's recommendations. Following scanning, array data processing, normalization (Rank Invariant), and analysis was performed with Illumina BeadStudio software. ). Array values shown on the Tables are the mean of the 3 replicates for each sample.

**Quantitative RT-PCR.** Ten micrograms of total RNA from each sample were treated with RNase-free DNase according to the manufacturer's protocol (Ambion, Austin TX). First-strand DNA synthesis was synthesized using 4μg of DNA-free RNA with Oligo-dT primers and Superscript II reverse transcriptase according to the manufacturer's

recommendations (Invitrogen). PCR reactions (25µl) contained 1µl of cDNA template, 12.5µl 2x SYBR Green I Master Mix (Bio-Rad, Hercules, CA) and 250nM forward and reverse primer. Reactions were carried out in a Bio-Rad iCycler. The cycling conditions were as follows: polymerase activation 10 minutes at 95°C and 40 cycles at 95°C for 20 sec, 60°C for 20 sec, and 72°C for 20 sec followed by melt curve analysis. All reactions were performed in biological triplicates and experiments contained –RT reactions for each sample and a no template control for each primer set. PCR efficiencies for all primer pairs were determined by performing a standard curve of 3 serial 1:4 dilutions of HT-22 cDNA. The expression of the gene of interest was normalized in parallel reactions with TATA binding protein (TBP). This gene was not regulated by corticosterone treatment in HT-22 cells. Primer pairs for housekeeping gene (TBP) and Dbp were obtained from Primer Bank (Wang and Seed 2003). Primer pairs for all the other genes used were designed using Primer3 platform. Changes in gene expression levels were quantitated with efficiency correction using the following mathematical formula, ratio=(Etarget) $^{\Delta CPtarget}$/(Eref) $^{\Delta CPref}$ (Abbreviations: E, efficiency; CP, threshold crossing point; target, target gene; ref, reference gene (Pfaffl, Lange et al. 2003) which accounts for differences in primer efficiencies. Data represent the average of three biological replicates.

Real-Time RT-PCR primers:

| Gene | Forward Primer | Reverse Primer | Amplicon size | Melt Temp (Tm) |
|---|---|---|---|---|
| Per2 | gtgcgatgatgattcgtgag | aaaagacacaagcagtcacacaa | 106bp | 60/60 C |
| Per3 | gtacaccggcaatctctgct | caacacacacttttcttaccaattc | 137bp | 60/59 C |
| NR1D1 | gggctctggtgctgaaga | gtccacccggaaggacag | 125bp | 60/61 C |
| TEF | aaagaggccaggagaaaagc | cccaacttccctccataaca | 143bp | 60/60 |

| | | | | |
|---|---|---|---|---|
| Dbp | cctgatcccgctgatctcg | caggcacctggactttcctt | 133bp | 62/62 |
| TBP | agaacaatccagactagcagca | gggaacttcacatcacagctc | 120bp | 59/60 C |

**Identification of Differentially Expressed Genes.** Differentially expressed genes were identified by comparing corticosterone-treated values to vehicle-treated values within each cell line using the following criteria: 1) differential expression required a Diff. score of ± 17 (p-value = 0.02) and 2) differential expression required at least a ± 1.5 fold (or greater) change in expression. We found that genes with average intensity values of less than 100 were highly variable within the triplicate samples for that group due to their very low expression. Therefore, genes were only included in the analysis if their average expression intensity values were greater than 100 in at least one of the comparison groups.

**Gene Ontology Classification of Differentially Expressed Genes.** The Gene Ontology Tree Machine website (http://bioinfo.vanderbilt.edu/gotm) was used assign gene ontology (GO) terms to each gene and to determine which categories of genes are overrepresented in the data. The GoMiner website (http://discover.nci.nih.gov/gominer/) was used in combination with VennMaster software (http://www.informatik.uni-ulm.de/ni/staff/HKestler/vennm/doc.html) to also assign GO terms to the differentially expressed genes and display the overrepresented categories in Venn diagram form.

***Results:***

Microarray Data:

Biological replicates (n=3) were used to generate triplicate RNA samples and biotinylated aRNA probe sets for each condition: HT-22 Parent cells treated with vehicle alone (P0), 1nM corticosterone (P1), or 100nm corticosterone (P100), and HT-22/MR16 cells treated as above (M0, M1, and M100). Each Illumina BeadChip array contained ~42,000 genes and each gene was represented with, on average, a 30 fold redundancy to provide a measure of internal variability. Signal intensity values are reported in arbitrary units and ranged from -50 to ~80,000 for all the genes on the array. As expected, most of the highly expressed genes (signal intensity values of =/> 10,000 on the array) were involved in general cellular biosynthetic processes, including many ribosomal and structural genes. As a measure of array integrity, scatter plots were generated from all the genes on the arrays comparing the vehicle-treated control groups to the corticosterone-treated groups within each cell line. Figure 4-1 depicts the scatter plots of P0 vs. P1, P0 vs. P100, M0 vs. M1, and M0 vs. M100. A visual inspection of the data reveals that the average signal intensity values for most of the genes are equivalent between each treatment group suggesting that there were no significant abnormalities in the RNA or probe integrity between groups. The points above or below the main line indicate genes whose expression is altered by the treatment; as predicted, the majority of genes with altered expression are present in the P0 to P100 and M0 to M100 comparisons.

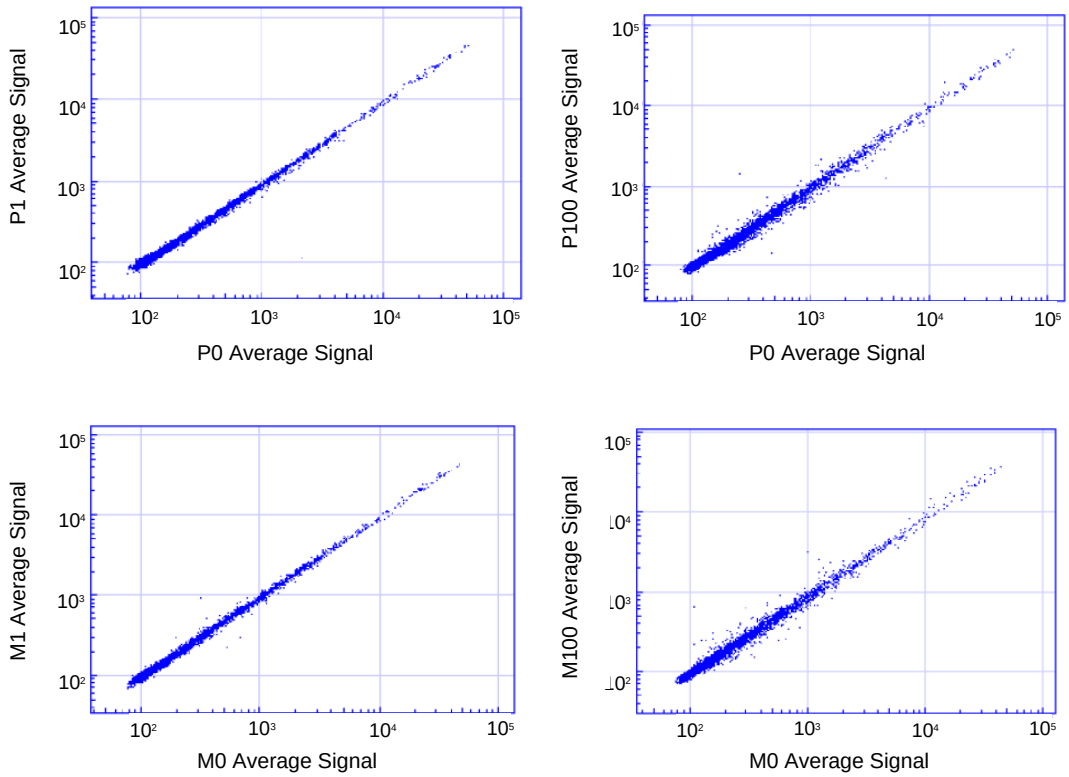



**Figure 4-1 Array integrity is equivalent between samples.**

Scatter plots containing the average intensity values of all the genes on the array are compared between vehicle treated control cells on the x-axis and the respective treatment groups on the y-axis. Visual inspection of the graphs suggests that there are no major differences in sample integrity between groups. P0=HT-22 Parent vehicle control, P1=HT-22 Parent 1nM corticosterone, P100=HT-22 Parent 100nM corticosterone, M0=HT-22 MR16 vehicle control, M1= HT-22 MR16 1nM cortiocsterone, and M100=HT-22 MR16 100nM corticosterone.

*Corticosterone-responsive genes activated or repressed by GR occupation:*

Using the criteria outlined in the Materials and Methods section (Diff. score>17 and change >1.5 fold), differentially expressed genes were identified in the HT-22 Parent cell line in response to treatment with 1nM or 100nM corticosterone when compared to vehicle alone. In response to 1nM corticosterone, only 4 genes were differentially regulated, all of which were repressed. These included two unknown genes, 2600005O03Rik (-1.9 fold change in expression, Diff. score -39.04) and LOC234640 (1.5 fold, Diff. score -22.94), and two known genes, the ubiquitin specific protease Usp1 (-1.5 fold, Diff. score -22.79), and the Arf-like GTPase Arl10b (-1.5 fold, Diff. score 27.29). As HT-22 cells do not express MR, we did not expect to see many genes whose expression was altered at 1nM corticosterone, significantly below the Kd for GR.

However, as predicted, 252 genes were up or down-regulated in the HT-22 Parent cell line in response to 100nM corticosterone. Of the 252 regulated genes, 193 were identified as known genes and the other 59 are largely represented by cDNAs from the RIKEN Mouse Encyclopedia Project. Seventy-nine percent of these genes were down-regulated and 21% were up-regulated. Table 4-1 lists the 252 genes that were present at significantly different levels in the P100 group with respect to the vehicle-treated P0 group, and Table 4-2 lists the 10 genes that are induced or repressed to the greatest extent. As this cell line only expresses GR, the regulation of these genes can be said to be GR-specific.

To identify classes of regulated genes and place them into functional categories, gene ontology (GO) terms are assigned to each gene. Classes of genes that are overrepresented in the data can be identified by generating ratios of the observed number

**Table 4-1 Corticosterone responsive genes of HT-22 Parent cells activated or repressed following GR induction.**

| 0nM Cort | 100nM Cort | Fold Change | Diff Score | Accession | Gene Symbol |
|---|---|---|---|---|---|
| 388.1 | 258.5 | -1.5 | -25.798 | NM_021446 | 0610007P14Rik |
| 307.2 | 206.7 | -1.5 | -27.882 | NM_026746.1 | 1110014D18Rik |
| 646.1 | 394.4 | -1.6 | -41.631 | NM_025391 | 1110017C15Rik |
| 462.1 | 286.5 | -1.6 | -41.205 | | 1110048P06Rik |
| 1019.8 | 655.9 | -1.6 | -21.52 | XM_147036.4 | 1190002N15Rik |
| 163.4 | 248.5 | 1.5 | 23.38 | AK078433 | 1200008A14Rik |
| 248.3 | 169.4 | -1.5 | -17.951 | NM_026424.2 | 1500041J02Rik |
| 93.4 | 141.8 | 1.5 | 17.124 | | 1600002O04Rik |
| 123.9 | 194.1 | 1.6 | 27.959 | NM_138581.1 | 1700088E04Rik |
| 216.5 | 112.7 | -1.9 | -24.604 | NM_029682.2 | 1700095N21Rik |
| 510.3 | 328.9 | -1.6 | -34.933 | AK008437 | 2010204K13Rik |
| 241.5 | 162.4 | -1.5 | -20.265 | NM_133839.1 | 2010311D03Rik |
| 431.3 | 278.7 | -1.5 | -17.408 | NM_025917.1 | 2010315L10Rik |
| 105.7 | 205.9 | 1.9 | 48.629 | XM_489155 | 2310001H17Rik |
| 244.6 | 167.6 | -1.5 | -22.329 | XM_128102.2 | 2410187C16Rik |
| 186.3 | 103.5 | -1.8 | -27.27 | NM_026453.1 | 2600016B03Rik |
| 362.5 | 232.3 | -1.6 | -37.803 | NM_019836 | 2610024G14Rik |
| 493 | 296.7 | -1.7 | -38.809 | NM_146130 | 2610510D13Rik |
| 264.2 | 179.5 | -1.5 | -25.262 | NM_026528.1 | 2700060E02Rik |
| 132.8 | 241 | 1.8 | 22.326 | | 2900042O04Rik |
| 249.4 | 144 | -1.7 | -28.921 | XM_126172 | 3110040D16Rik |
| 1938.9 | 1286.9 | -1.5 | -22.067 | XM_203376.1 | 3732413I11Rik |
| 614.3 | 934.7 | 1.5 | 21.292 | AK029315 | 4832420L08Rik |
| 251.7 | 161.6 | -1.6 | -19.327 | NM_172499.1 | 4931419K03Rik |
| 113.5 | 63.1 | -1.8 | -17.266 | BC018281 | 6030411K04Rik |
| 170.8 | 97.3 | -1.8 | -25.307 | | 9930009M05Rik |
| 329.5 | 225.6 | -1.5 | -28.09 | | 9930105H17Rik |
| 233.1 | 151.7 | -1.5 | -18.063 | AK044233 | A930002H24Rik |
| 280.2 | 176.5 | -1.6 | -31.041 | NM_133237.1 | AB023957 |
| 199.9 | 126.5 | -1.6 | -20.132 | NM_013853.1 | Abcf2 |
| 231.9 | 146.4 | -1.6 | -28.239 | NM_013853.1 | Abcf2 |
| 148.7 | 251.2 | 1.7 | 45.732 | NM_019811.2 | Acas2 |
| 248.1 | 145.1 | -1.7 | -22.782 | NM_013777.1 | Akr1c12 |
| 252.2 | 164.1 | -1.5 | -28.35 | NM_019705.2 | AL033326 |
| 282.8 | 161.9 | -1.7 | -44.334 | NM_021505.1 | Anapc5 |
| 1727.4 | 892.8 | -1.9 | -53.585 | NM_013468.2 | Ankrd1 |
| 362.8 | 532.9 | 1.5 | 27.902 | NM_013471 | Anxa4 |
| 120.2 | 72.7 | -1.7 | -17.235 | NM_025545.2 | Aptx |
| 134.6 | 37.6 | -3.6 | -50.369 | NM_009701.1 | Aqp5 |
| 784.4 | 490.2 | -1.6 | -36.53 | NM_007480.1 | Arf5 |
| 284 | 444.6 | 1.6 | 26.43 | NM_007489.1 | Arntl |
| 1295.7 | 836.5 | -1.5 | -35.709 | NM_019767.1 | Arpc1a |
| 266.3 | 136.6 | -1.9 | -54.083 | NM_012055.1 | Asns |
| 1964.3 | 1200.1 | -1.6 | -33.035 | NM_009716 | Atf4 |
| 336 | 219.3 | -1.5 | -22.264 | NM_026195.1 | Atic |
| 308.1 | 207.7 | -1.5 | -28.831 | NM_027439 | Atp6ap2 |

| 0nM Cort | 100nM Cort | Fold Change | Diff Score | Accession | Gene Symbol |
|---|---|---|---|---|---|
| 159.4 | 93.7 | -1.7 | -21.542 | NM_027439.2 | Atp6ap2 |
| 119.2 | 66.3 | -1.8 | -20.705 | NM_027439.2 | Atp6ap2 |
| 166.2 | 102.8 | -1.6 | -18.127 | NM_007517.1 | Aup1 |
| 395 | 254.4 | -1.6 | -37.161 | NM_198609 | BC003885 |
| 454.8 | 294 | -1.5 | -19.795 | NM_033562.2 | BC005682 |
| 243.5 | 426.6 | 1.8 | 31.344 | NM_146237 | BC024537 |
| 251.9 | 164.6 | -1.5 | -23.6 | NM_172149 | Bnip1 |
| 232.1 | 148.4 | -1.6 | -23.227 | NM_012047.1 | Brd7 |
| 553.7 | 254.4 | -2.2 | -85.486 | NM_007569.1 | Btg1 |
| 512.9 | 235.4 | -2.2 | -81.036 | NM_007569.1 | Btg1 |
| 4057.8 | 2608.5 | -1.6 | -29.063 | NM_007573.1 | C1qbp |
| 244.6 | 165.8 | -1.5 | -20.496 | NM_026561.2 | C330027I04Rik |
| 1041.2 | 2139.1 | 2.1 | 21.008 |  | C920004C08Rik |
| 246.4 | 169.6 | -1.5 | -20.588 | NM_133926.1 | Camk1 |
| 133.5 | 76.5 | -1.7 | -17.575 | NM_007664.1 | Cdh2 |
| 2850.7 | 1776.9 | -1.6 | -41.887 | NM_009870.2 | Cdk4 |
| 1211.9 | 743.2 | -1.6 | -60.444 | NM_009870.2 | Cdk4 |
| 136.2 | 239.7 | 1.8 | 34.553 | NM_009893.1 | Chrd |
| 212.7 | 102.8 | -2.1 | -49.03 | NM_023850.1 | Chst1 |
| 1049.1 | 523.6 | -2.0 | -24.487 | NM_007739.1 | Col8a1 |
| 164.2 | 343.2 | 2.1 | 37.381 | NM_007752 | Cp |
| 1924.8 | 2835 | 1.5 | 46.425 | NM_009964.1 | Cryab |
| 132.8 | 73.3 | -1.8 | -23.735 | NM_009968.1 | Cryz |
| 215.3 | 144.4 | -1.5 | -21.003 | NM_139117 | Csda |
| 115.3 | 236.5 | 2.1 | 18.856 | NM_019389.1 | Cspg2 |
| 491.6 | 306.5 | -1.6 | -47.889 | NM_016748.1 | Ctps |
| 128.8 | 46.8 | -2.8 | -27.293 | NM_010516.1 | Cyr61 |
| 54.9 | 258.4 | 4.7 | 138.754 |  | D15Bwg0759e |
| 807.9 | 1270.4 | 1.6 | 40.612 |  | D15Mit260 |
| 5968.1 | 10897.8 | 1.8 | 101.066 | NM_033075.2 | D17H6S56E-5 |
| 1218.4 | 2062 | 1.7 | 72.231 | NM_033075.2 | D17H6S56E-5 |
| 189.9 | 122.8 | -1.5 | -19.54 | NM_175514.1 | D430039N05Rik |
| 411.4 | 41.9 | -9.8 | 244.639 | NM_016974.1 | Dbp |
| 504.7 | 319.4 | -1.6 | -24.64 | NM_201408 | Dhps |
| 274.2 | 162.7 | -1.7 | -34.503 | NM_019448.2 | Dnmt3l |
| 461.4 | 795.9 | 1.7 | 36.447 | NM_013642.1 | Dusp1 |
| 353.1 | 177.3 | -2.0 | -23.252 | NM_176933 | Dusp4 |
| 256.8 | 151.9 | -1.7 | -29.111 | NM_008748.1 | Dusp8 |
| 207.5 | 348.6 | 1.7 | 49.271 | NM_021469 | Dysf |
| 901.8 | 510 | -1.8 | -37.108 | NM_007907.1 | Eef2 |
| 332.4 | 229.1 | -1.5 | -18.39 | NM_145371.1 | Eif2b1 |
| 422.7 | 249.7 | -1.7 | -34.705 | NM_146200 | Eif3s8 |
| 572.4 | 386.4 | -1.5 | -25.419 | NM_133916.1 | Eif3s9 |
| 158.8 | 94.7 | -1.7 | -22.075 | XM_147230.1 | Eif4a2 |
| 1676 | 960.1 | -1.7 | -65.302 | NM_181582.2 | Eif5a |
| 1725.1 | 896.6 | -1.9 | -78.533 |  | Eif5a |
| 133.5 | 58.9 | -2.3 | -27.305 | NM_010139.2 | Epha2 |
| 208 | 103.5 | -2.0 | -28.677 | NM_007945.1 | Eps8 |
| 2818.9 | 1717.9 | -1.6 | -32.925 | NM_016903.2 | Esd |
| 499.4 | 315.2 | -1.6 | -36.703 | NM_145615.2 | Etfa |

| 0nM Cort | 100nM Cort | Fold Change | Diff Score | Accession | Gene Symbol |
|---|---|---|---|---|---|
| 187 | 87.1 | -2.1 | -35.862 | NM_025923.2 | Fancl |
| 135.8 | 218 | 1.6 | 17.907 | NM_134469 | Fdps |
| 1364 | 2441.1 | 1.8 | 18.274 | NM_010216.1 | Figf |
| 365.3 | 713.9 | 2.0 | 63.229 | NM_010220.2 | Fkbp5 |
| 271.3 | 178.4 | -1.5 | -19.4 | XM_127565.4 | Flnb |
| 400.6 | 250.5 | -1.6 | -42.449 | XM_129845.3 | Fn1 |
| 980 | 590.4 | -1.7 | -58.549 | XM_129845.3 | Fn1 |
| 815 | 441.8 | -1.8 | -17.208 | NM_008037.3 | Fosl2 |
| 120 | 64 | -1.9 | -22.662 | NM_011814.1 | Fxr2h |
| 132.8 | 79.1 | -1.7 | -19.628 | NM_013716.1 | G3bp |
| 145 | 93.7 | -1.5 | -17.1 | NM_175935.2 | G6pc3 |
| 2373.8 | 1540.8 | -1.5 | -28.244 | NM_007836.1 | Gadd45a |
| 67.6 | 115.6 | 1.7 | 17.106 | NM_053108 | Glrx1 |
| 263.2 | 162.6 | -1.6 | -32.527 | NM_010301.1 | Gna11 |
| 125.8 | 73.3 | -1.7 | -19.212 | NM_010309.1 | Gnas |
| 389.7 | 211.9 | -1.8 | -38.752 | NM_010309.1 | Gnas |
| 431.6 | 266.7 | -1.6 | -17.981 | NM_016739.2 | Gpiap1 |
| 311.7 | 201.9 | -1.5 | -33.32 | NM_153419.1 | Grwd1 |
| 675.9 | 463.7 | -1.5 | -22.729 | NM_025652.1 | Gtf3a |
| 233.5 | 147.8 | -1.6 | -29.081 | NM_022331.1 | Herpud1 |
| 946.1 | 622.1 | -1.5 | -33.103 | NM_182650.2 | Hnrpa2b1 |
| 284.7 | 176.5 | -1.6 | -35.939 | NM_025279.1 | Hnrpk |
| 770.7 | 497.3 | -1.5 | -49.771 | XM_355338.1 | Hoxd8 |
| 630.2 | 939.8 | 1.5 | 39.221 | NM_178162.2 | Hrbl |
| 196.5 | 110.1 | -1.8 | -28.883 | XM_147286.1 | Hs6st1 |
| 1110.6 | 624 | -1.8 | -19.358 | NM_010480 | Hspca |
| 6170.1 | 3545.1 | -1.7 | -38.64 | NM_008302.2 | Hspcb |
| 3337.8 | 1865.8 | -1.8 | -69.914 | NM_008302.2 | Hspcb |
| 417.1 | 237.7 | -1.8 | -31.09 | NM_010477.2 | Hspd1 |
| 141.2 | 280.4 | 2.0 | 60.755 | AK013239 | Idb2 |
| 1110.6 | 741.5 | -1.5 | -24.718 | NM_008323.1 | Idh3g |
| 347.7 | 104.8 | -3.3 | -109.15 | NM_010743.1 | Il1rl1 |
| 156.2 | 89.1 | -1.8 | -17.996 | NM_008363.1 | Irak1 |
| 207.6 | 111.9 | -1.9 | -24.455 | NM_013566.1 | Itgb7 |
| 311.7 | 193 | -1.6 | -21.8 | NM_010591.1 | Jun |
| 296.2 | 199.5 | -1.5 | -23.935 | NM_011317.2 | Khdrbs1 |
| 376.2 | 250.6 | -1.5 | -22.796 | NM_010637.1 | Klf4 |
| 174.2 | 76.8 | -2.3 | -18.7 | NM_010637 | Klf4 |
| 1550.3 | 875.6 | -1.8 | -21.593 | XM_111684.3 | LOC195357 |
| 315.7 | 179.6 | -1.8 | -36.17 | XM_137003.2 | LOC212399 |
| 198 | 123 | -1.6 | -18.419 | XM_123387.2 | LOC226036 |
| 312.5 | 125.8 | -2.5 | -32.234 | XM_205287.3 | LOC279653 |
| 859.1 | 520.1 | -1.7 | -45.775 | XM_203701.3 | LOC280487 |
| 250 | 105.1 | -2.4 | -67.084 | XM_207856.3 | LOC280487 |
| 1759.5 | 1109.6 | -1.6 | -61.062 | XM_147224.2 | LOC328703 |
| 4299.2 | 2713 | -1.6 | -37.554 | XM_287286.2 | LOC329750 |
| 826.4 | 566.9 | -1.5 | -24.216 | XM_354540.1 | LOC380634 |
| 1316.4 | 860.3 | -1.5 | -25.907 | XM_354663.1 | LOC380749 |
| 1226.8 | 801.1 | -1.5 | -26.87 | XM_354669.1 | LOC380756 |
| 256.6 | 171.3 | -1.5 | -20.839 | XM_355597.1 | LOC381632 |

| 0nM Cort | 100nM Cort | Fold Change | Diff Score | Accession | Gene Symbol |
|---|---|---|---|---|---|
| 184.4 | 109.7 | -1.7 | -22.199 | XM_359170.1 | LOC386324 |
| 1429.9 | 772 | -1.9 | -27.105 | XM_359287.1 | LOC386463 |
| 1894.3 | 3437.7 | 1.8 | 52.546 | NM_010741 | Ly6c |
| 265.8 | 388 | 1.5 | 35.692 | NM_011838.2 | Lynx1 |
| 1047.2 | 613.2 | -1.7 | -20.546 | NM_008549.1 | Man2a1 |
| 133.2 | 251.1 | 1.9 | 40.296 | NM_016693 | Map3k6 |
| 785.9 | 509.8 | -1.5 | -22.78 | NM_015806.2 | Mapk6 |
| 121.5 | 51.6 | -2.4 | -22.599 | NM_145569 | Mat2a |
| 514.1 | 312.5 | -1.6 | -41.701 | NM_013595.1 | Mbd3 |
| 342 | 221.1 | -1.5 | -17.535 | NM_019709.3 | Mbtps1 |
| 57.4 | 175.6 | 3.1 | 66.251 | NM_008597.2 | Mglap |
| 1158.2 | 2187.7 | 1.9 | 374.344 | NM_021462.2 | Mknk2 |
| 70.6 | 118.8 | 1.7 | 19.386 | NM_021462 | Mknk2 |
| 398.6 | 267.1 | -1.5 | -30.247 | NM_053159.2 | Mrpl3 |
| 850.5 | 568.3 | -1.5 | -19.893 | NM_026971.2 | Mrpl48 |
| 420.1 | 273.9 | -1.5 | -27.89 | NM_026971.2 | Mrpl48 |
| 1297.5 | 300.2 | -4.3 | -128.38 | NM_011954.2 | Mrpplf3 |
| 245.8 | 432.3 | 1.8 | 23.474 | NM_018857.1 | Msln |
| 149.3 | 90.7 | -1.6 | -19.374 | NM_010833.1 | Msn |
| 1591.3 | 4368 | 2.7 | 32.135 | NM_008630.1 | Mt2 |
| 577.4 | 381.6 | -1.5 | -37.151 | NM_019880.2 | Mtch1 |
| 230.5 | 131.4 | -1.8 | -27.138 | NM_016776.2 | Mybbp1a |
| 139.5 | 78.5 | -1.8 | -21.423 | NM_010849.2 | Myc |
| 137.6 | 66.4 | -2.1 | -31.769 | NM_025898.1 | Napa |
| 374 | 819.7 | 2.2 | 85.412 | NM_010875 | Ncam1 |
| 1169.7 | 782.4 | -1.5 | -20.124 | NM_026554.2 | Ncbp2 |
| 294.5 | 415.3 | 1.4 | 34.067 | AK052726 | Nfkb1 |
| 150.4 | 53.9 | -2.8 | -52.109 | NM_013609.1 | Ngfb |
| 200.2 | 120.4 | -1.7 | -21.027 | NM_025623.1 | Nipsnap3b |
| 109.9 | 56.5 | -1.9 | -21.408 | NM_025623.1 | Nipsnap3b |
| 144 | 214.9 | 1.5 | 21.451 | NM_010924.1 | Nnmt |
| 1205.4 | 661.6 | -1.8 | -35.315 | NM_024193.1 | Nol5a |
| 185.4 | 71.6 | -2.6 | -58.353 | NM_145434.1 | Nr1d1 |
| 59.1 | 239.2 | 4.0 | 33.62 | NM_009108.1 | Nr1h4 |
| 183.6 | 119.7 | -1.5 | -20.853 | NM_026012.1 | Nradd |
| 231.6 | 129.4 | -1.8 | -23.236 | NM_011956.2 | Nubp2 |
| 132.2 | 75.8 | -1.7 | -20.296 | NM_175360.2 | Obfc1 |
| 649.6 | 390.4 | -1.7 | -17.917 | NM_011858 | Odz4 |
| 112.3 | 180.7 | 1.6 | 26.999 | NM_033321 | P2rx5 |
| 737.8 | 425.5 | -1.7 | -35.056 | NM_011119.1 | Pa2g4 |
| 178.5 | 972.1 | 5.4 | 143.525 | NM_013743.1 | Pdk4 |
| 52.5 | 246.1 | 4.7 | 93.662 | NM_011065.2 | Per1 |
| 140.1 | 60.7 | -2.3 | -38.186 | NM_011067 | Per3 |
| 872.9 | 485.8 | -1.8 | -30.364 | NM_009344.1 | Phlda1 |
| 4846 | 3309.5 | -1.5 | -21.844 | NM_133779.1 | Pigt |
| 432.1 | 205 | -2.1 | -22.859 | NM_011117 | Plec1 |
| 126.7 | 75.5 | -1.7 | -17.769 | NM_023900.1 | Plekhj1 |
| 4987.5 | 1393.2 | -3.6 | -69.344 | NM_031191 | Plf |
| 835.4 | 221.9 | -3.8 | -113.39 | NM_031191.1 | Plf |
| 1847.1 | 424.6 | -4.4 | -114.69 | NM_011118.1 | Plf2 |

| 0nM Cort | 100nM Cort | Fold Change | Diff Score | Accession | Gene Symbol |
|---|---|---|---|---|---|
| 2667.4 | 1601 | -1.7 | -43.495 | NM_152804.1 | Plk2 |
| 2785.8 | 1775.7 | -1.6 | -43.509 | NM_031868.1 | Ppp1ca |
| 1178.6 | 808.2 | -1.5 | -31.737 | NM_016891.2 | Ppp2r1a |
| 534.9 | 1519.6 | 2.8 | 79.579 | NM_054077.2 | Prelp |
| 338.8 | 223.6 | -1.5 | -21.192 | NM_011158.2 | Prkar2b |
| 154.9 | 88.6 | -1.7 | -25.937 | NM_080469.2 | Prnpip1 |
| 108.3 | 51.6 | -2.1 | -21.884 | NM_019564.1 | Prss11 |
| 1325.2 | 738.6 | -1.8 | -65.213 | XM_128721.2 | Prss15 |
| 900.1 | 620.2 | -1.5 | -22.734 | NM_011965 | Psma1 |
| 429.7 | 255 | -1.7 | -19.511 | NM_011875 | Psmd13 |
| 1311.1 | 741.4 | -1.8 | -24.052 | NM_010817.1 | Psmd7 |
| 782.2 | 511.4 | -1.5 | -42.547 | NM_011190.2 | Psme2 |
| 314.4 | 163 | -1.9 | -24.428 | NM_008974.2 | Ptp4a2 |
| 201.8 | 117.3 | -1.7 | -17.035 | NM_009010.2 | Rad23a |
| 196.9 | 134.1 | -1.5 | -17.696 | NM_019930.1 | Ranbp9 |
| 3506.1 | 2221.5 | -1.6 | -28.211 | NM_009031.2 | Rbbp7 |
| 3743.3 | 2314.3 | -1.6 | -24.605 | NM_009031.2 | Rbbp7 |
| 205.5 | 101.8 | -2.0 | -20.965 | NM_009034 | Rbp2 |
| 245.9 | 380.1 | 1.5 | 21.663 | NM_023275.1 | Rhoj |
| 210.7 | 325.4 | 1.5 | 39.087 | NM_177620.2 | Rin3 |
| 166.8 | 92.2 | -1.8 | -17.274 | NM_024242.2 | Riok1 |
| 136.7 | 74.2 | -1.8 | -20.53 | NM_194054.1 | Rtn4 |
| 293.7 | 172.7 | -1.7 | -42.541 | NM_019685.1 | Ruvbl1 |
| 167.8 | 104.1 | -1.6 | -22.917 | NM_025614 | Rwdd1 |
| 118.3 | 184.9 | 1.6 | 26.293 | NM_020568.1 | S3-12 |
| 122.5 | 223 | 1.8 | 24.141 | NM_011315 | Saa3 |
| 238.6 | 148.2 | -1.6 | -21.894 | NM_025516.2 | Sdbcag84 |
| 278.3 | 499.3 | 1.8 | 32.036 | XM_125538.4 | Sesn1 |
| 470.4 | 291 | -1.6 | -27.013 | NM_009186 | Sfrs10 |
| 758.1 | 447.6 | -1.7 | -19.425 | NM_009159 | Sfrs5 |
| 1638.5 | 2704.7 | 1.7 | 40.524 | NM_011361.1 | Sgk |
| 116.2 | 63.1 | -1.8 | -18.472 | NM_172507.2 | Sh3bgrl2 |
| 275.6 | 411.6 | 1.5 | 17.885 | NM_011893 | Sh3bp2 |
| 257.6 | 138.9 | -1.9 | -26.415 | NM_021389.3 | Sh3kbp1 |
| 196.5 | 124.6 | -1.6 | -23.381 | NM_020618.3 | Smarce1 |
| 185.3 | 108.4 | -1.7 | -18.247 | NM_019710.1 | Smc1l1 |
| 83.7 | 151.5 | 1.8 | 17.278 | NM_021491.2 | Smpd3 |
| 193.5 | 126.9 | -1.5 | -22.177 | NM_021535.2 | Smu1 |
| 285.4 | 193.1 | -1.5 | -23.551 | NM_009224.2 | Snrp70 |
| 326.8 | 219.7 | -1.5 | -30.197 | NM_015782.2 | Snrpa |
| 493.1 | 301.4 | -1.6 | -48.579 | NM_015782.2 | Snrpa |
| 488.4 | 294.3 | -1.7 | -51.315 | NM_015782.2 | Snrpa |
| 371.6 | 624.4 | 1.7 | 29.975 | NM_011451.1 | Sphk1 |
| 895.6 | 590.1 | -1.5 | -24.716 | NM_009272.2 | Srm |
| 781.1 | 494.4 | -1.6 | -53.248 | NM_019879.1 | Suclg1 |
| 244.7 | 126.6 | -1.9 | -24.243 | NM_028958.2 | Taf7l |
| 362.4 | 238.8 | -1.5 | -20.796 | NM_013686 | Tcp1 |
| 202.2 | 328.6 | 1.6 | 51.149 | NM_011567.1 | Tead4 |
| 1208.2 | 424.9 | -2.8 | -128.27 | NM_017376.2 | Tef |
| 140.8 | 85.1 | -1.7 | -20.506 | NM_011570.2 | Tes |

| 0nM Cort | 100nM Cort | Fold Change | Diff Score | Accession | Gene Symbol |
|---|---|---|---|---|---|
| 2659.1 | 1722.4 | -1.5 | -27.907 | NM_011595.1 | Timp3 |
| 184 | 112.8 | -1.6 | -18.018 | NM_019793.2 | Tm4sf8 |
| 1507 | 910.7 | -1.7 | -17.676 | XM_122498.1 | Tm7sf1 |
| 3272.5 | 2028.7 | -1.6 | -43.248 | NM_013749.1 | Tnfrsf12a |
| 472.3 | 290.5 | -1.6 | -39.531 | NM_023680 | Tnfrsf22 |
| 182.7 | 79.9 | -2.3 | -30.451 | NM_007987.1 | Tnfrsf6 |
| 182.1 | 107.2 | -1.7 | -26.396 | NM_016788 | Tnk2 |
| 1372.1 | 933.6 | -1.5 | -19.069 | NM_011623.1 | Top2a |
| 274.4 | 153.2 | -1.8 | -30.374 | XM_134274.3 | Tpm4 |
| 309.5 | 194.3 | -1.6 | -21.583 | NM_175093.1 | Trib3 |
| 331.6 | 484.2 | 1.5 | 31.996 | NM_144899.2 | Tsrc1 |
| 2365.4 | 1403.7 | -1.7 | -49.195 | NM_011655.2 | Tubb5 |
| 228 | 131.3 | -1.7 | -36.365 | NM_009454.2 | Ube2e3 |
| 406.5 | 234 | -1.7 | -33.679 | NM_019748.1 | Uble1a |
| 303.9 | 194.8 | -1.6 | -26.899 | NM_019562.1 | Uchl5 |
| 340.4 | 206.3 | -1.7 | -43.135 | NM_009471.1 | Umps |
| 253.6 | 372.2 | 1.5 | 38.283 | NM_009500 | Vav2 |
| 1761.7 | 1143.5 | -1.5 | -23.018 | NM_018865.1 | Wisp1 |
| 325.9 | 191.8 | -1.7 | -34.324 | NM_133756.2 | Xab1 |
| 273.3 | 181.2 | -1.5 | -21.523 | XM_125902.4 | Xpot |
| 399.8 | 260.6 | -1.5 | -22.264 | NM_026570.1 | Yeats4 |
| 992.2 | 642.9 | -1.5 | -36.674 | NM_011739 | Ywhaq |
| 209.5 | 129.6 | -1.6 | -18.611 | NM_080855.1 | Zcchc14 |
| 336.9 | 226 | -1.5 | -22.836 | NM_145600.1 | Zfp330 |
| 243.6 | 167 | -1.5 | -18.149 | NM_207302 | Zranb1 |
| 973.3 | 1925.1 | 2.0 | 61.439 | X06328.1 | |
| 388 | 577.8 | 1.5 | 52.182 | AK049469.1 | |
| 370.5 | 251.6 | -1.5 | -24.768 | XM_127742.4 | |
| 201.8 | 113.3 | -1.8 | -31.586 | AF411517.1 | |
| 762.3 | 406.1 | -1.9 | -26.635 | AK077302.1 | |
| 122.7 | 48.8 | -2.5 | -35.957 | XM_358429.1 | |

Listed from left to right, the average signal intensity of each gene in the vehicle treated control (0nM Cort), 100nM corticosterone treated group (100nM Cort), the fold change in expression level from basal to corticosterone treatment, the significance value in Diff. score (Diff. 17 is equivalent to p=0.02, larger Diff. scores are lower p-values), the accession number, and the gene symbol for each gene regulated by corticosterone in HT-22 Parent cells.

**Table 4-2 Ten genes most highly induced or repressed in response to corticosterone in HT-22 Parent and HT-22/MR16 cells.**

| P0 - P100nM | | | | M0 - M1nM | | | | M0 - M100nM | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Fold Change** | **Diff Score** | **Accession** | **Gene Symbol** | **Fold Change** | **Diff Score** | **Accession** | **Gene Symbol** | **Fold Change** | **Diff Score** | **Accession** | **Gene Symbol** |
| 5.4 | 143.525 | NM_013743.1 | Pdk4 | 4.1 | 141.5 | NM_008491.1 | Lcn2 | 8.2 | 18.551 | NM_008491.1 | Lcn2 |
| 4.7 | 138.754 | | D15Bwg0759e | 3.8 | 46.5 | NM_008581.1 | Mela | 6.0 | 18.931 | NM_008581.1 | Mela |
| 4.7 | 93.662 | NM_011065.2 | Per1 | 2.8 | 40.3 | NM_007752 | Cp | 5.6 | 122.373 | NM_011065.2 | Per1 |
| 4.0 | 33.62 | NM_009108.1 | Nr1h4 | 2.8 | 120.8 | NM_054077.2 | Prelp | 5.2 | 43.735 | | D15Bwg0759e |
| 3.1 | 66.251 | NM_008597.2 | Mglap | 2.6 | 23.9 | NM_011065.2 | Per1 | 4.8 | 112.369 | NM_011451.1 | Sphk1 |
| 2.8 | 79.579 | NM_054077.2 | Prelp | 2.6 | 76.0 | NM_011451.1 | Sphk1 | 4.7 | 115.757 | NM_007752 | Cp |
| 2.7 | 32.135 | NM_008630.1 | Mt2 | 2.5 | 21.8 | NM_009504.2 | Vdr | 3.7 | 31.973 | NM_010286.2 | Dsip1 |
| 2.2 | 85.412 | NM_010875 | Ncam1 | 2.4 | 30.1 | | D15Bwg0759e | 3.5 | 53.705 | NM_008630.1 | Mt2 |
| 2.1 | 37.381 | NM_007752 | Cp | 2.4 | 58.3 | NM_009108.1 | Nr1h4 | 3.3 | 27.946 | NM_013743.1 | Pdk4 |
| 2.1 | 18.856 | NM_019389.1 | Cspg2 | 2.0 | 50.6 | NM_009504.2 | Vdr | 3.0 | 31.507 | NM_054077.2 | Prelp |
| -2.6 | -58.353 | NM_145434.1 | Nr1d1 | -1.6 | -34.9 | NM_031191 | Plf | -3.8 | -93.06 | NM_011066.1 | Per2 |
| -2.8 | -27.293 | NM_010516.1 | Cyr61 | -1.6 | -24.0 | NM_009344.1 | Phlda1 | -3.9 | -51.159 | XM_148699.3 | Crebbp |
| -2.8 | -52.109 | NM_013609.1 | Ngfb | -1.6 | -25.8 | NM_011066.1 | Per2 | -4.0 | -60.684 | NM_198416.1 | Zzz3 |
| -2.8 | -128.27 | NM_017376.2 | Tef | -1.7 | -17.1 | NM_080428 | Fbxw7 | -4.1 | -120.138 | NM_145434.1 | Nr1d1 |
| -3.3 | -109.15 | NM_010743.1 | Il1rl1 | -1.7 | -20.2 | NM_009263.1 | Spp1 | -4.2 | -104.484 | NM_031191.1 | Plf |
| -3.6 | -50.369 | NM_009701.1 | Aqp5 | -1.8 | -19.1 | NM_012019.2 | Pdcd8 | -4.5 | -62.212 | NM_007661.2 | Cdc2l2 |
| -3.6 | -69.344 | NM_031191 | Plf | -1.9 | -43.7 | NM_145434.1 | Nr1d1 | -4.6 | -33.761 | AK017756 | Hcapg-pending |
| -4.3 | -128.38 | NM_011954.2 | Mrpplf3 | -1.9 | -88.6 | NM_017376.2 | Tef | -5.1 | -27.083 | NM_178930 | Gbf1 |
| -4.4 | -114.69 | NM_011118.1 | Plf2 | -2.0 | -38.8 | NM_011067 | Per3 | -6.6 | -68.88 | NM_007887.1 | Dub1 |
| -9.8 | -244.63 | NM_016974.1 | Dbp | -2.8 | -155.1 | NM_016974.1 | Dbp | -16.1 | -370.442 | NM_016974.1 | Dbp |

Listed from left to right for each treatment group, the fold change in expression level from basal to corticosterone treatment, the significance value in Diff. score (Diff. 17 is equivalent to p=0.02, larger Diff. scores are lower p-values), the accession number, and the gene symbol for each gene.

of genes regulated in a category versus the expected number of genes for that category. To assign GO terms to the list of changed genes in the P100 group we used the Gene Ontology Tree Machine (http://bioinfo.vanderbilt.edu/gotm) website (GOTM). GOTM was able to assign GO terms for 164 of the 252 genes submitted (65%). Of the 164 GO-termed genes that were regulated by corticosterone in the HT-22 Parent cell line, many different classes of genes were identified with the largest fraction falling into the categories of cellular physiological process, metabolism, and regulation of physiological process (Figure 4-2A). Categories of genes that are classified as overrepresented in the data fall under broad cellular and physiological processes, such as cellular metabolism and cell growth, and include genes specifically involved in cell adhesion, regulation of transcription, intracellular protein transport, and intracellular signaling (phosphatases, kinases, and transcription factors). Figure 4-2B represents a Venn diagram of overrepresented categories of genes that have changed in the P100 group relative to the vehicle-treated control as determined by the GoMiner Ontogeny Software Program. This analysis also indicates that, by and large, the gene categories most affected by corticosterone treatment relate to cell growth. Several other categories were identified in this analysis, including genes involved in the epigenetic regulation of gene expression, nuclear cytoplasmic transport and 6 genes involved in circadian rhythmic processes. All together, there is a clear effect of corticosterone on expression of genes involved in neuronal metabolism and cell growth in the HT-22 hippocampal cell line.

A.

QuickTime™ and a
TIFF (Uncompressed) decompressor
are needed to see this picture.

B.

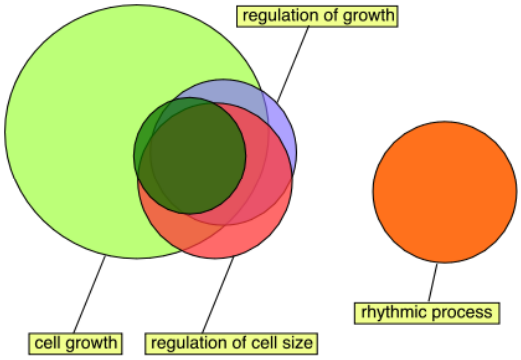



**Figure 4-2 Gene Ontology of genes regulated by 100nm corticosterone in HT-22 Parent cells.**

A) Quantitation of genes that sort into different functional categories under the ontology of Biological Process as determined by GOTM software. Categories in red denote an overrepresentation of regulated genes relative to the expected number of genes within that category. B) Vennmaster diagram depicting overlapping functional categories of genes that were also determined to be overrepresented in the data as determined by GoMiner and VennMaster software.

*Corticosterone-responsive genes activated or repressed by MR occupation:*

The HT-22/MR16 clonal cell line expresses significant levels of both MR and GR. However, treatment of these cells with 1 nM corticosterone would be expected to activate MR, with only a small effect on GR, due to their significant differences in corticosterone binding affinities. A comparison of the gene expression profiles from vehicle-treated HT-22/MR16 (M0) with the 1nM-treated cells (M1) resulted in the identification of 41 genes whose expression was significantly different between groups. Fifty percent of these genes were up-regulated and 50% of them were down-regulated (Table 4-3) and Table 4-2 lists the 10 genes that are induced and repressed to the greatest extent. Of the 41 genes, 37 were identified as known genes (90%) and the other four were identified in the RIKEN Mouse Encyclopedia Project. GO terms were assigned to 38 of the 41 genes allowing for functional classification of gene classes. The genes regulated by MR largely fit into the same functional classes as GR-regulated genes (Figure 4-3). Categories of genes that were identified as overrepresented in the data include a number of proteins with kinase activity involved in cellular physiological processes such as cell adhesion, and most prominently, genes involved in circadian rhythm.

*Corticosterone-responsive genes activated or repressed by MR and GR occupation at 100nM corticosterone:*

Comparison of the gene expression profiles from vehicle-treated HT-22/MR16 (M0) and 100nM corticosterone-treated cells (M100) resulted in the identification of 476 genes that were significantly different between groups (Table 4-4) and Table 4-2 lists the

**Table 4-3 Corticosterone responsive genes activated or repressed following MR induction in HT-22/MR16 cells treated with 1nM corticosterone.**

| 0nM Cort | 1nM Cort | Fold Change | Diff Score | Accession | Gene Symbol |
|---|---|---|---|---|---|
| | 107.2 | -1.6 | -23.2 | AK077748 | 5730594E01Rik |
| 191.1 | 317.9 | 1.7 | 17.7 | NM_007489.1 | Arntl |
| 350.3 | 207.5 | -1.7 | -31.2 | AK046455 | B230387C07Rik |
| 506.6 | 757.6 | 1.5 | 25.9 | NM_009778.1 | C3 |
| 112.1 | 61.2 | -1.8 | -18.5 | AK021175 | C330004K01Rik |
| 263 | 166.1 | -1.6 | -26.8 | NM_023850.1 | Chst1 |
| 955 | 611.3 | -1.6 | -27.0 | NM_007739.1 | Col8a1 |
| 354.5 | 242.1 | -1.5 | -24.1 | NM_007739 | Col8a1 |
| 58 | 162.4 | 2.8 | 40.3 | NM_007752 | Cp |
| 124.9 | 210.7 | 1.7 | 27.6 | NM_019389.1 | Cspg2 |
| 3071 | 2171.9 | -1.4 | -22.9 | NM_008176.1 | Cxcl1 |
| 181.3 | 116.2 | -1.6 | -18.4 | NM_010516.1 | Cyr61 |
| 53.3 | 126.9 | 2.4 | 30.1 | | D15Bwg0759e |
| 554.1 | 195.9 | -2.8 | -155.1 | NM_016974.1 | Dbp |
| 254.3 | 172.6 | -1.5 | -18.7 | NM_009468.1 | Dpysl3 |
| 137.2 | 82.9 | -1.7 | -17.1 | NM_080428 | Fbxw7 |
| 311.3 | 457.3 | 1.5 | 27.9 | NM_010220.2 | Fkbp5 |
| 253.8 | 390.4 | 1.5 | 17.2 | NM_020583.4 | Isg20 |
| 267.5 | 1086.1 | 4.1 | 141.5 | NM_008491.1 | Lcn2 |
| 31.6 | 119.2 | 3.8 | 46.5 | NM_008581.1 | Mela |
| 1166.1 | 1984.5 | 1.7 | 30.8 | NM_008630.1 | Mt2 |
| 194 | 293.8 | 1.5 | 18.4 | NM_010875.2 | Ncam1 |
| 703.2 | 479.5 | -1.5 | -21.5 | NM_008688.2 | Nfic |
| 239.6 | 127.8 | -1.9 | -43.7 | NM_145434.1 | Nr1d1 |
| 104.2 | 248 | 2.4 | 58.3 | NM_009108.1 | Nr1h4 |
| 103.2 | 163.8 | 1.6 | 18.9 | NM_033321 | P2rx5 |
| 188.2 | 106.5 | -1.8 | -19.1 | NM_012019.2 | Pdcd8 |
| 316.4 | 527.9 | 1.7 | 21.3 | NM_013743.1 | Pdk4 |
| 52 | 137.4 | 2.6 | 23.9 | NM_011065.2 | Per1 |
| 198.9 | 122.1 | -1.6 | -25.8 | NM_011066.1 | Per2 |
| 176.6 | 86.8 | -2.0 | -38.8 | NM_011067 | Per3 |
| 957.7 | 590 | -1.6 | -24.0 | NM_009344.1 | Phlda1 |
| 187.9 | 118.3 | -1.6 | -17.4 | NM_011113.2 | Plaur |
| 3858.1 | 2377.3 | -1.6 | -34.9 | NM_031191 | Plf |
| 476.7 | 1329.3 | 2.8 | 120.8 | NM_054077.2 | Prelp |
| 1481 | 2002.3 | 1.4 | 19.1 | NM_011361.1 | Sgk |
| 1348.4 | 907.3 | -1.5 | -17.6 | NM_019654.2 | Socs5 |
| 102.1 | 269.3 | 2.6 | 76.0 | NM_011451.1 | Sphk1 |
| 1368.5 | 824.6 | -1.7 | -20.2 | NM_009263.1 | Spp1 |
| 1549 | 806 | -1.9 | -88.6 | NM_017376.2 | Tef |
| 322.5 | 645.9 | 2.0 | 50.6 | NM_009504.2 | Vdr |
| 46.3 | 115 | 2.5 | 21.8 | NM_009504.2 | Vdr |
| 154.2 | 256.8 | 1.7 | 31.1 | NM_016873.1 | Wisp2 |

Listed from left to right, the average signal intensity of each gene in the vehicle treated control (0nM Cort), 1nM corticosterone treated group (1nM Cort), the fold change in expression level from basal to corticosterone treatment, the significance value in Diff. score (Diff. 17 is equivalent to p=0.02, larger Diff. scores are lower p-values), the accession number, and the gene symbol for each gene regulated in HT-22/MR16 cells.

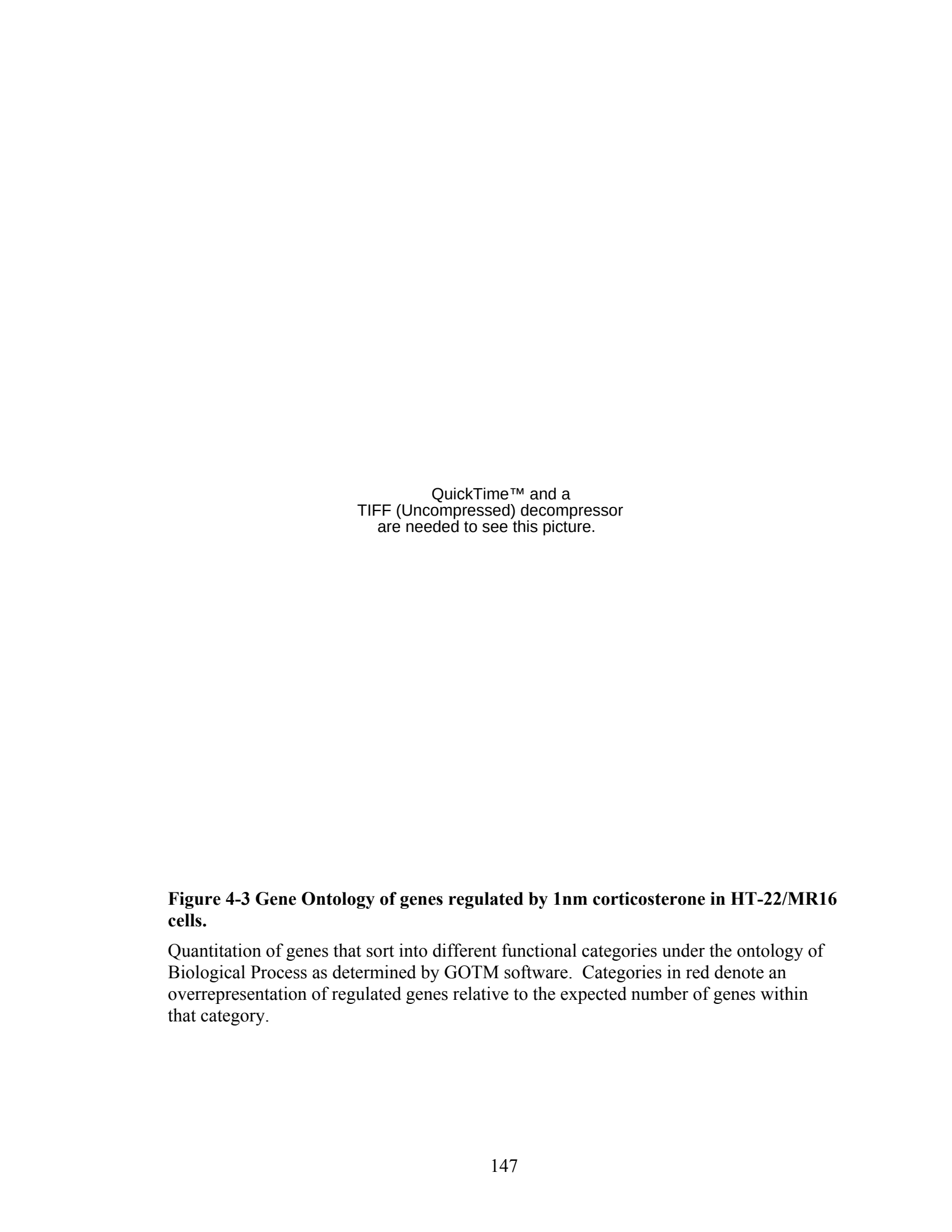



**Figure 4-3 Gene Ontology of genes regulated by 1nm corticosterone in HT-22/MR16 cells.**

Quantitation of genes that sort into different functional categories under the ontology of Biological Process as determined by GOTM software. Categories in red denote an overrepresentation of regulated genes relative to the expected number of genes within that category.

10 genes that were induced or repressed to the greatest extent. Of these 476 genes, 89% were down-regulated and 11% were up-regulated. In this list of genes, 311 (65%) were identified as known genes and the other 35% were identified in the RIKEN Mouse Encyclopedia Project. GO terms could be linked with 254 of the 476 genes (53%). As with the other two groups, the greatest number of genes regulated by MR and GR fit into the functional classes of cellular physiological processes and metabolism (Figure 4-4A). Categories of genes that are overrepresented in the data include genes involved in cell differentiation and cell growth, circadian rhythm, and a large group of 23 genes that are involved in programmed cell death. Figure 4-4B represents a Venn diagram of overrepresented categories of genes that have changed in the M100 group relative to the vehicle-treated control as determined by GoMiner ontogeny analysis. This analysis also highlights the overrepresentation of genes involved in circadian rhythm, as well as the positive regulation of cell proliferation, and identifies a subset of the genes (8 of 23 genes) in the programmed cell death category as being involved in the negative regulation of cell death.

*Comparison of corticosteroid-regulated genes between groups:*

To identify genes that may be specifically regulated by GR homodimers, MR homodimers, and/or GR/MR heterodimers, the lists of regulated genes from the P0-P100, M0-M1, and M0-M100 groups were compared. As the P0-P1 list only contained 4 genes it was not included in this analysis. Figure 4-5 represents a Venn diagram of the three lists of regulated genes and the number of genes that overlap between each group, and Table 4-5 contains the lists of genes that are represented in each category. Of the 252 GR

**Table 4-4 Corticosterone responsive genes activated or repressed following MR and GR induction with 100nM corticosterone in HT-22/MR16 cells.**

| 0nM Cort | 100nM Cort | Fold Change | Diff Score | Accession | Gene Symbol |
|---|---|---|---|---|---|
| 243.2 | 150.7 | -1.6 | -21.158 | | 0610007N19Rik |
| 402.3 | 191.7 | -2.1 | -78.588 | NM_025793.1 | 0610008N23Rik |
| 311 | 183 | -1.7 | -20.746 | NM_025339.2 | 0610027O18Rik |
| 263.1 | 138 | -1.9 | -25.843 | NM_172401.2 | 1110057K04Rik |
| 888.1 | 569.8 | -1.6 | -43.593 | XM_147036.4 | 1190002N15Rik |
| 600.7 | 314.1 | -1.9 | -49.456 | NM_025822.2 | 1200013F24Rik |
| 324.7 | 725.6 | 2.2 | 18.248 | NM_133753.1 | 1300002F13Rik |
| 790.1 | 326.7 | -2.4 | -70.78 | XM_132343.3 | 1500011J06Rik |
| 228 | 121.9 | -1.9 | -17.63 | XM_356257.1 | 1500041I23Rik |
| 268.9 | 162.2 | -1.7 | -22.081 | NM_026424.2 | 1500041J02Rik |
| 1152 | 714.7 | -1.6 | -39.585 | | 1500041O16Rik |
| 57.6 | 144.6 | 2.5 | 38.021 | AK006897 | 1700065O13Rik |
| 94.5 | 195.1 | 2.1 | 38.277 | XM_283372.2 | 1700110N18Rik |
| 106.9 | 187 | 1.7 | 26.24 | NM_026931.1 | 1810011O10Rik |
| 210.7 | 139.7 | -1.5 | -20.431 | AK007762 | 1810043M20Rik |
| 287.8 | 162.8 | -1.8 | -25.496 | | 2010205O06Rik |
| 450.4 | 220.3 | -2.0 | -59.912 | NM_177157.2 | 2010323F13Rik |
| 559.8 | 308.9 | -1.8 | -65.221 | NM_177157.2 | 2010323F13Rik |
| 133.2 | 62.9 | -2.1 | -28.99 | AK008639 | 2200005K02Rik |
| 653.2 | 374.8 | -1.7 | -18.274 | | 2210008N01Rik |
| 139.7 | 40.3 | -3.5 | -46.766 | | 2210401K01Rik |
| 1311.1 | 798 | -1.6 | -59.752 | XM_204313.2 | 2300009A05Rik |
| 116.5 | 189.2 | 1.6 | 21.834 | XM_489155 | 2310001H17Rik |
| 1056.2 | 717.4 | -1.5 | -33.713 | NM_028800 | 2310004N11Rik |
| 257.1 | 495.4 | 1.9 | 59.479 | NM_027992 | 2310036D22Rik |
| 141 | 281.2 | 2.0 | 44.025 | NM_027992.1 | 2310036D22Rik |
| 415.8 | 69.3 | -6.0 | -115.16 | AK009823 | 2310045K21Rik |
| 93.7 | 186.2 | 2.0 | 31.306 | | 2310051E17Rik |
| 530.4 | 242.1 | -2.2 | -74.663 | NM_133784.1 | 2310058J06Rik |
| 732.4 | 489.7 | -1.5 | -27.866 | NM_028013.1 | 2310067E08Rik |
| 1356.5 | 785.8 | -1.7 | -39.615 | XM_204283.3 | 2410003B16Rik |
| 1312.8 | 898.4 | -1.5 | -23.015 | NM_023203.1 | 2410015N17Rik |
| 1238.8 | 787.7 | -1.6 | -34.933 | NM_030241.2 | 2410195B05Rik |
| 954.3 | 624.5 | -1.5 | -28.056 | NM_030241.2 | 2410195B05Rik |
| 248.8 | 162.9 | -1.5 | -18.446 | AK010848 | 2500002A22Rik |
| 320.3 | 166.1 | -1.9 | -50.05 | NM_172947.1 | 2600001B17Rik |
| 816.2 | 471.7 | -1.7 | -30.318 | NM_172947.1 | 2600001B17Rik |
| 753.7 | 471.5 | -1.6 | -36.294 | NM_028244.1 | 2600005C20Rik |
| 172.7 | 41.6 | -4.2 | -31.488 | AK011206 | 2600014C01Rik |
| 755.3 | 438.9 | -1.7 | -43.658 | | 2610002F03Rik |
| 171.3 | 92.5 | -1.9 | -19.643 | AK011403 | 2610015J01Rik |
| 8695.5 | 5608.9 | -1.6 | -19.777 | | 2610019E17Rik |
| 421.7 | 265.8 | -1.6 | -35.164 | AK011472 | 2610019N13Rik |
| 488.2 | 267.3 | -1.8 | -49.161 | XM_110750 | 2610030H06Rik |
| 118.7 | 55.5 | -2.1 | -19.742 | AK011690 | 2610036C07Rik |
| 337.7 | 211.5 | -1.6 | -33.407 | NM_175364.2 | 2610208E05Rik |

| 0nM Cort | 100nM Cort | Fold Change | Diff Score | Accession | Gene Symbol |
|---|---|---|---|---|---|
| 420.2 | 286.6 | -1.5 | -28.301 | NM_028818.1 | 2610511M17Rik |
| 272.5 | 114.2 | -2.4 | -29.217 | | 2610528A11Rik |
| 802.7 | 497 | -1.6 | -23.723 | | 2700023E23Rik |
| 1337.9 | 605.6 | -2.2 | -56.841 | AK019219 | 2700079K05Rik |
| 245.7 | 138.2 | -1.8 | -25.546 | | 2700083E18Rik |
| 1356.4 | 930.4 | -1.5 | -23.448 | NM_026025 | 2700088M22Rik |
| 168.3 | 110.6 | -1.5 | -17.297 | NM_026028.1 | 2700091N06Rik |
| 207.2 | 135.3 | -1.5 | -20.899 | NM_025479.2 | 2810021B07Rik |
| 169.9 | 104.4 | -1.6 | -21.212 | | 2810402E24Rik |
| 253.6 | 117.6 | -2.2 | -31.698 | AK013109 | 2810417K24Rik |
| 237.1 | 131.8 | -1.8 | -19.331 | | 2810468A05Rik |
| 294.6 | 107.9 | -2.7 | -41.321 | XM_132966.3 | 2810474O19Rik |
| 200.3 | 67.2 | -3.0 | -36.391 | AK013732 | 2900060F21Rik |
| 178.5 | 92.2 | -1.9 | -25.181 | XM_127780.4 | 3110050K21Rik |
| 3458.4 | 1798.2 | -1.9 | -56.272 | NM_026313.1 | 3300001P08Rik |
| 633.6 | 359.6 | -1.8 | -37.169 | XM_196166 | 3300005D01Rik |
| 138.2 | 79 | -1.7 | -19.243 | | 4633401G24Rik |
| 322.9 | 218.2 | -1.5 | -18.137 | | 4833438C02Rik |
| 311 | 60.8 | -5.1 | -85.987 | AK029517 | 4921511I23Rik |
| 284.6 | 80.3 | -3.5 | -90.747 | XM_130287.5 | 4930432B04Rik |
| 108.3 | 47.3 | -2.3 | -25.69 | XM_147531.1 | 4930527B16Rik |
| 722.2 | 493.4 | -1.5 | -28.125 | | 4930563B10Rik |
| 115.5 | 64.7 | -1.8 | -18.043 | | 4930568E02Rik |
| 327.7 | 213.2 | -1.5 | -28.768 | NM_029404.1 | 4932409F11Rik |
| 273.3 | 184.1 | -1.5 | -23.359 | XM_356109.1 | 4933407C03Rik |
| 177.7 | 79.4 | -2.2 | -17.499 | AK077171 | 4933417E08Rik |
| 1203.8 | 664.8 | -1.8 | -23.936 | XM_127929.3 | 4933421G18Rik |
| 145.1 | 86 | -1.7 | -20.256 | | 4933431K14Rik |
| 398.2 | 250.3 | -1.6 | -35.709 | XM_127301.2 | 5033430I15Rik |
| 325.6 | 124.1 | -2.6 | -83.653 | | 5430405H02Rik |
| 116.7 | 55.1 | -2.1 | -24.053 | | 5430411C19Rik |
| 272.7 | 178.4 | -1.5 | -18.837 | | 5530400B01Rik |
| 473.8 | 171.1 | -2.8 | -117.92 | AK077637 | 5730494J16Rik |
| 1441 | 781.4 | -1.8 | -86.452 | NM_025690 | 5730555F13Rik |
| 422.6 | 147.5 | -2.9 | -52.394 | AK030766 | 5730577G12Rik |
| 176.7 | 42.1 | -4.2 | -57.572 | AK077748 | 5730594E01Rik |
| 280.4 | 169.1 | -1.7 | -26.396 | XM_488874 | 5830406C17Rik |
| 311.3 | 167 | -1.9 | -50.164 | NM_026583.2 | 5830415L20Rik |
| 515.5 | 322.7 | -1.6 | -42.761 | NM_176921.2 | 6030419C18Rik |
| 744.9 | 1210.1 | 1.6 | 20.864 | | 6330414G02Rik |
| 131.6 | 49.7 | -2.6 | -24.319 | AK033615 | 9130202B12Rik |
| 701.2 | 426.7 | -1.6 | -26.755 | NM_177206 | 9230101K24Rik |
| 226.3 | 83.1 | -2.7 | -44.055 | XM_110546.2 | 9230115A19Rik |
| 228.9 | 136.7 | -1.7 | -20.258 | AK020369 | 9330132O05Rik |
| 856 | 560.9 | -1.5 | -40.775 | XM_355205 | 9430025M21Rik |
| 247.7 | 126.9 | -2.0 | -34.491 | AK020610 | 9530055J05Rik |
| 1351.5 | 748.3 | -1.8 | -51.994 | XM_489200 | 9530064J02 |
| 232.2 | 133.1 | -1.7 | -25.764 | AK079342 | 9630032J03Rik |
| 327.8 | 213.3 | -1.5 | -20.119 | | 9930105H17Rik |

| 0nM Cort | 100nM Cort | Fold Change | Diff Score | Accession | Gene Symbol |
|---|---|---|---|---|---|
| 345.6 | 183.7 | -1.9 | -35.393 | AK040788 | A530025E09Rik |
| 1722.7 | 1065.4 | -1.6 | -24.843 | AK079972 | A530030G15Rik |
| 327.3 | 224.3 | -1.5 | -24.411 | AK041191 | A530089A20Rik |
| 146.1 | 46.8 | -3.1 | -53.077 | AK041811 | A630040A01Rik |
| 305.5 | 204.1 | -1.5 | -21.972 | NM_177857 | A930010I20Rik |
| 167.6 | 75.5 | -2.2 | -41.499 | NM_177232 | A930033H14Rik |
| 117.3 | 34.8 | -3.4 | -39.475 | AK080758 | A930101O15Rik |
| 301.7 | 139.9 | -2.2 | -61.289 | NM_133237.1 | AB023957 |
| 309.1 | 172.6 | -1.8 | -24.33 | NM_007382 | Acadm |
| 6654.4 | 4476.8 | -1.5 | -25.923 | NM_009636.1 | Aebp1 |
| 1134.5 | 740.7 | -1.5 | -18.748 | NM_019914.2 | AI839562 |
| 1071.5 | 613.6 | -1.7 | -24.934 | NM_198616.1 | AI842788 |
| 386.8 | 179.5 | -2.2 | -40.632 | NM_194462.1 | Akap9 |
| 155.9 | 78.8 | -2.0 | -27.925 | NM_011785.2 | Akt3 |
| 3604.2 | 1821.9 | -2.0 | -35.957 | NM_009656.1 | Aldh2 |
| 81.2 | 143.6 | 1.8 | 21.023 | NM_009667 | Ampd3 |
| 193.5 | 81.2 | -2.4 | -54.131 | NM_011923.2 | Angptl2 |
| 743.5 | 316.5 | -2.3 | -30.527 | NM_020581.1 | Angptl4 |
| 258.6 | 77.5 | -3.3 | -29.497 |  | Ankhd1 |
| 5043.6 | 3261.5 | -1.5 | -19.807 | NM_007472.1 | Aqp1 |
| 445.7 | 223 | -2.0 | -41.632 | NM_153800.2 | Arhgap22 |
| 105.2 | 52.6 | -2.0 | -20.217 | NM_145996.2 | Arid5a |
| 191.1 | 336.7 | 1.8 | 40.414 | NM_007489.1 | Arntl |
| 663.7 | 454.2 | -1.5 | -25.087 | NM_145822.1 | Ase1 |
| 391.2 | 253.6 | -1.5 | -19.747 | NM_138679.2 | Ash1l |
| 590.2 | 383.4 | -1.5 | -21.636 | NM_007496.1 | Atbf1 |
| 1648 | 1089.6 | -1.5 | -21.708 | NM_007496.1 | Atbf1 |
| 199.9 | 115.1 | -1.7 | -23.154 | NM_025983.2 | Atp5e |
| 212.3 | 127.6 | -1.7 | -22.596 | NM_007507 | Atp5k |
| 557.8 | 344.7 | -1.6 | -24.064 | NM_134034.1 | AW011752 |
| 1942.2 | 1229.3 | -1.6 | -44.857 | NM_144879.1 | B130052G07Rik |
| 360.7 | 148.1 | -2.4 | -34.191 | AK045321 | B130065G19Rik |
| 328.5 | 190.9 | -1.7 | -39.203 | XM_130148.1 | B230208H17Rik |
| 415.1 | 167.5 | -2.5 | -57.226 | AK046451 | B230386D16Rik |
| 350.3 | 148.2 | -2.4 | -62.665 | AK046455 | B230387C07Rik |
| 251.5 | 125.9 | -2.0 | -24.785 | NM_178764.2 | B930006L02Rik |
| 786.1 | 526.1 | -1.5 | -33.845 | AK046967 | B930008G03Rik |
| 158 | 241.8 | 1.5 | 21.989 | NM_173002.1 | BC003332 |
| 299.2 | 185.8 | -1.6 | -22.367 | NM_145430.1 | BC017647 |
| 646.7 | 424.8 | -1.5 | -29.424 | NM_175002.1 | BC025076 |
| 474.1 | 230.7 | -2.1 | -66.198 | NM_198167.1 | BC026370 |
| 248.9 | 168.4 | -1.5 | -19.446 | NM_145943.1 | BC031781 |
| 807.1 | 548.3 | -1.5 | -35.772 | XM_109956 | BC037006 |
| 276.3 | 177.2 | -1.6 | -18.1 | NM_172761.1 | BC052066 |
| 270.8 | 120 | -2.3 | -27.44 | NM_183208.2 | BC065120 |
| 952.3 | 625.8 | -1.5 | -28.204 | NM_009740.1 | Bcl10 |
| 342.8 | 188.8 | -1.8 | -51.118 | NM_009744.2 | Bcl6 |
| 1296 | 813.6 | -1.6 | -29.977 | NM_011498.2 | Bhlhb2 |
| 884 | 476.3 | -1.9 | -67.129 | NM_020508.2 | Brd4 |

| 0nM Cort | 100nM Cort | Fold Change | Diff Score | Accession | Gene Symbol |
|---|---|---|---|---|---|
| 207.2 | 97 | -2.1 | -27.809 | NM_133195.1 | Brunol4 |
| 910.1 | 540.5 | -1.7 | -20.211 | NM_133195 | Brunol4 |
| 362.6 | 178.6 | -2.0 | -34.08 | | C030034I22Rik |
| 211.3 | 108 | -2.0 | -36.421 | AK048061 | C130032J12Rik |
| 139.5 | 71.1 | -2.0 | -24.678 | | C130080N23Rik |
| 163.3 | 104.9 | -1.6 | -18.115 | XM_110478.3 | C330013J21Rik |
| 375.2 | 177.6 | -2.1 | -55.004 | NM_139304.1 | C430014D17Rik |
| 169 | 91 | -1.9 | -29.894 | | C730026J16 |
| 144.2 | 71.4 | -2.0 | -27.373 | NM_153547.2 | C77032 |
| 2942.1 | 2012 | -1.5 | -18.848 | NM_009794.1 | Capn2 |
| 307.3 | 167.3 | -1.8 | -25.987 | NM_009810.1 | Casp3 |
| 425.3 | 212.6 | -2.0 | -47.052 | NM_007631.1 | Ccnd1 |
| 1346.5 | 779.4 | -1.7 | -37.103 | NM_007631.1 | Ccnd1 |
| 569.3 | 333.4 | -1.7 | -53.853 | NM_009829.2 | Ccnd2 |
| 1598.1 | 1038.1 | -1.5 | -17.988 | NM_009831.1 | Ccng1 |
| 158.3 | 55 | -2.9 | -52.572 | NM_054042.2 | Cd164l1 |
| 1080.6 | 552 | -2.0 | -47.885 | NM_133654.1 | Cd34 |
| 1033.3 | 648.9 | -1.6 | -51.422 | AK045226 | Cd44 |
| 171.3 | 86.1 | -2.0 | -34.736 | NM_007658 | Cdc25a |
| 136 | 30.2 | -4.5 | -62.212 | NM_007661.2 | Cdc2l2 |
| 1783.3 | 1009.6 | -1.8 | -37.182 | NM_007669.2 | Cdkn1a |
| 1497.7 | 1024.4 | -1.5 | -24.487 | NM_007669.2 | Cdkn1a |
| 287.8 | 143 | -2.0 | -17.282 | AK021188 | Chd1 |
| 118.1 | 61.8 | -1.9 | -17.392 | NM_145979.1 | Chd4 |
| 129.7 | 261.3 | 2.0 | 26.237 | NM_009893.1 | Chrd |
| 263 | 92.4 | -2.8 | -92.66 | NM_023850.1 | Chst1 |
| 379.2 | 165.1 | -2.3 | -81.342 | NM_175554.3 | Clspn |
| 827 | 489.8 | -1.7 | -59.871 | NM_007725.1 | Cnn2 |
| 512.4 | 227.5 | -2.3 | -59.407 | NM_016877.2 | Cnot4 |
| 480.8 | 292.9 | -1.6 | -30.767 | NM_053007.1 | Cntf |
| 326.7 | 165.3 | -2.0 | -52.736 | NM_007743.1 | Col1a2 |
| 955 | 384.2 | -2.5 | -81.213 | NM_007739.1 | Col8a1 |
| 354.5 | 157.4 | -2.3 | -83.061 | NM_007739 | Col8a1 |
| 2631.5 | 1443.8 | -1.8 | -38.537 | NM_011803.1 | Copeb |
| 174.6 | 103.2 | -1.7 | -24.621 | NM_025379.1 | Cox7b |
| 58 | 270.3 | 4.7 | 115.757 | NM_007752 | Cp |
| 348.4 | 88.8 | -3.9 | -51.159 | XM_148699.3 | Crebbp |
| 171.9 | 53.6 | -3.2 | -32.405 | XM_148699.3 | Crebbp |
| 192.7 | 121.5 | -1.6 | -22.413 | NM_133679.1 | Cryzl1 |
| 2083.7 | 1250.3 | -1.7 | -29.182 | NM_007778.1 | Csf1 |
| 302.4 | 145.1 | -2.1 | -53.275 | AK031617 | Csnk2a1-rs3 |
| 124.9 | 256.1 | 2.1 | 18.669 | NM_019389.1 | Cspg2 |
| 849.5 | 567.2 | -1.5 | -18.772 | NM_145529.1 | Cstf3 |
| 869.1 | 582.5 | -1.5 | -29.009 | NM_198683.1 | Cugbp1 |
| 181.3 | 62 | -2.9 | -56.311 | NM_010516.1 | Cyr61 |
| 172.2 | 68.1 | -2.5 | -44.666 | NM_010516.1 | Cyr61 |
| 477.3 | 279 | -1.7 | -36.952 | NM_146155.2 | D030015G18Rik |

| 0nM Cort | 100nM Cort | Fold Change | Diff Score | Accession | Gene Symbol |
|---|---|---|---|---|---|
| 1009.7 | 546 | -1.8 | -51.13 | NM_025602.1 | D10Ertd718e |
| 101.3 | 52.7 | -1.9 | -17.946 | AK051470 | D130051G04Rik |
| 483.1 | 783.2 | 1.6 | 43.384 | AK051669 | D130063H01Rik |
| 53.3 | 274.5 | 5.2 | 43.735 | | D15Bwg0759e |
| 263 | 156.5 | -1.7 | -24.966 | NM_023063.1 | D15Ertd366e |
| 606.5 | 1079.7 | 1.8 | 60.028 | | D15Mit260 |
| 161.2 | 78.7 | -2.0 | -30.645 | NM_029648.3 | D19Ertd737e |
| 1155.1 | 762.7 | -1.5 | -26.477 | XM_129886.4 | D1Bwg1363e |
| 268.7 | 181.1 | -1.5 | -23.057 | XM_128090.5 | D330037H05Rik |
| 350.4 | 234.3 | -1.5 | -26.842 | NM_201372.1 | D630044F24Rik |
| 452.4 | 299.4 | -1.5 | -22.985 | | D730003I15Rik |
| 370.4 | 196 | -1.9 | -25.452 | | D730044K07Rik |
| 554.1 | 34.5 | -16.1 | -370.44 | NM_016974.1 | Dbp |
| 464 | 310.8 | -1.5 | -20.324 | NM_016765 | Ddah2 |
| 2093.8 | 1419.2 | -1.5 | -22.708 | NM_010026.1 | Ddef1 |
| 1291.7 | 515 | -2.5 | -163.98 | AK012125 | Ddx21 |
| 501.9 | 254.3 | -2.0 | -48.775 | NM_020494.2 | Ddx24 |
| 1021.6 | 402.4 | -2.5 | -159.47 | NM_007841.2 | Ddx6 |
| 619.4 | 245.5 | -2.5 | -32.941 | NM_025900.1 | Dek |
| 252.1 | 102.5 | -2.5 | -50.047 | XM_147318.1 | Dfna5h |
| 560.8 | 379.9 | -1.5 | -26.609 | NM_019670.1 | Diap3 |
| 223.3 | 144.8 | -1.5 | -20.354 | NM_019448.2 | Dnmt3l |
| 84.7 | 166.1 | 2.0 | 17.653 | NM_010070.3 | Dok1 |
| 82.5 | 183.1 | 2.2 | 35.535 | NM_010070.3 | Dok1 |
| 2772.8 | 1813.7 | -1.5 | -22.193 | XM_130951.1 | Dpm3 |
| 31.2 | 115.1 | 3.7 | 31.973 | NM_010286.2 | Dsip1 |
| 134.4 | 20.5 | -6.6 | -68.88 | NM_007887.1 | Dub1 |
| 412.9 | 837.7 | 2.0 | 24.271 | NM_013642.1 | Dusp1 |
| 325.1 | 137 | -2.4 | -71.353 | NM_176933.3 | Dusp4 |
| 206 | 128.5 | -1.6 | -20.591 | NM_008748.1 | Dusp8 |
| 51.5 | 105.9 | 2.1 | 20.158 | AK086999 | E030019D07Rik |
| 485.8 | 331.4 | -1.5 | -19.423 | NM_153791.1 | E030034P13Rik |
| 8686.1 | 5669 | -1.5 | -34.804 | AK053583 | E130112E08Rik |
| 206.9 | 133.5 | -1.5 | -22.101 | XM_127250 | E2f3 |
| 173.8 | 99.9 | -1.7 | -26.298 | NM_177133.1 | E330018D03Rik |
| 174.2 | 97.1 | -1.8 | -23.499 | AK088068 | E430003D02Rik |
| 258.9 | 130 | -2.0 | -26.841 | | E430024C06Rik |
| 1389.1 | 953.4 | -1.5 | -37.515 | NM_011816.2 | E430034L04Rik |
| 572.3 | 392.5 | -1.5 | -26.773 | NM_175540 | Eda2r |
| 793.4 | 541.2 | -1.5 | -34.998 | NM_010336 | Edg2 |
| 1167.2 | 788.2 | -1.5 | -32.172 | NM_021474.2 | Efemp2 |
| 2866.2 | 1893.9 | -1.5 | -45.838 | NM_198303 | Eif5b |
| 192.3 | 97.6 | -2.0 | -30.909 | NM_033612.1 | Ela1 |
| 175.9 | 60 | -2.9 | -61.615 | NM_023502.1 | Elf2 |
| 115.2 | 63.1 | -1.8 | -18.891 | NM_133918.1 | Emilin1 |
| 556.6 | 343.5 | -1.6 | -31.801 | XM_127139.5 | Eml1 |
| 199.5 | 129.6 | -1.5 | -18.575 | NM_199466.1 | Eml4 |
| 7581 | 4570.5 | -1.7 | -36.473 | NM_010128.3 | Emp1 |
| 4534.4 | 2684.6 | -1.7 | -52.792 | NM_010129 | Emp3 |

| 0nM Cort | 100nM Cort | Fold Change | Diff Score | Accession | Gene Symbol |
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| 161.4 | 78.2 | -2.1 | -35.178 | AK020248 | Enah |
| 147.2 | 64.6 | -2.3 | -35.788 | NM_199016.1 | Enpp4 |
| 171 | 97.5 | -1.8 | -25.62 | NM_010139.2 | Epha2 |
| 130.3 | 76.3 | -1.7 | -18.637 | XM_204072.3 | Ephb2 |
| 2666.5 | 1698.7 | -1.6 | -24.336 | NM_016903.2 | Esd |
| 135.4 | 70.1 | -1.9 | -25.465 | NM_008815 | Etv4 |
| 2022.6 | 1333.3 | -1.5 | -31.314 | NM_025644.3 | Exosc1 |
| 586.9 | 227.3 | -2.6 | -48.159 | NM_007971.1 | Ezh2 |
| 296.2 | 169.2 | -1.8 | -25.135 | AK089303 | F730003H07Rik |
| 4626.5 | 2840.7 | -1.6 | -42.33 | NM_007992.1 | Fbln2 |
| 137.2 | 37.7 | -3.6 | -41.693 | NM_080428 | Fbxw7 |
| 341.3 | 182.4 | -1.9 | -33.002 | NM_080428.2 | Fbxw7 |
| 176.9 | 101 | -1.8 | -21.824 | AK008093 | Fibp |
| 311.3 | 697.9 | 2.2 | 24.552 | NM_010220.2 | Fkbp5 |
| 70.8 | 137.2 | 1.9 | 24.257 | XM_288949.2 | Fmnl2 |
| 170.2 | 62.6 | -2.7 | -29.839 | NM_010235.1 | Fosl1 |
| 395 | 213.4 | -1.9 | -41.318 | NM_008655.1 | Gadd45b |
| 200.2 | 126.2 | -1.6 | -17.345 | NM_013814.2 | Galnt1 |
| 111.8 | 22 | -5.1 | -27.083 | NM_178930 | Gbf1 |
| 675.2 | 440.1 | -1.5 | -29.46 | NM_019683.2 | Gbif |
| 524.2 | 304 | -1.7 | -47.442 | NM_019832.2 | Gkap1 |
| 2471.8 | 1409.2 | -1.8 | -51.035 | NM_028608.1 | Glipr1 |
| 873.1 | 597.7 | -1.5 | -17.711 | NM_023505.1 | Glrx2 |
| 247.4 | 145.1 | -1.7 | -19.581 | NM_010320.2 | Gng8 |
| 584.9 | 370.7 | -1.6 | -42.923 | NM_027681.1 | Gnpda2 |
| 422.9 | 210.2 | -2.0 | -47.243 | NM_133852.1 | Golga2 |
| 346.9 | 147 | -2.4 | -32.57 | NM_175193.2 | Golph4 |
| 2872 | 1877.7 | -1.5 | -35.939 | NM_016696.1 | Gpc1 |
| 183.2 | 71.7 | -2.6 | -33.914 | NM_177346.1 | Gpr149 |
| 474.7 | 257 | -1.8 | -28.658 | AK012646 | Grb10 |
| 52 | 119.4 | 2.3 | 23.346 | BC060377 | Gsn |
| 1127.9 | 664.9 | -1.7 | -48.383 | NM_008197.2 | H1f0 |
| 190.9 | 118.8 | -1.6 | -19.041 | NM_013819.1 | H2-M3 |
| 135.9 | 29.7 | -4.6 | -33.761 | AK017756 | Hcapg-pending |
| 494.8 | 336.6 | -1.5 | -17.968 | NM_020045.1 | Hirip5 |
| 279.7 | 158.9 | -1.8 | -40.911 | NM_178187.2 | Hist1h2ae |
| 35916.3 | 24285.5 | -1.5 | -29.178 | NM_178185.1 | Hist1h2ao |
| 1073.1 | 696.1 | -1.5 | -45.791 | NM_010465.1 | Hoxc6 |
| 425.2 | 281.5 | -1.5 | -24.351 | NM_010472.1 | Hrb |
| 163.9 | 103.2 | -1.6 | -18.523 | XM_147286.1 | Hs6st1 |
| 814.1 | 522.6 | -1.6 | -43.084 | NM_133804.1 | Hspa5bp1 |
| 479.2 | 324.1 | -1.5 | -31.008 | NM_133804.1 | Hspa5bp1 |
| 1627.2 | 925.3 | -1.8 | -31.073 |  | Hspg2 |
| 784.2 | 518.1 | -1.5 | -30.108 | NM_031156.1 | Ide |
| 893.4 | 585.6 | -1.5 | -23.198 | NM_010517.2 | Igfbp4 |
| 608 | 414.8 | -1.5 | -23.465 | NM_010517.2 | Igfbp4 |
| 2092.4 | 1440 | -1.5 | -22.993 | NM_008344.1 | Igfbp6 |
| 2726 | 1593.1 | -1.7 | -68.716 | NM_016721.1 | Iqgap1 |
| 1149.2 | 768.4 | -1.5 | -24.301 | NM_016721 | Iqgap1 |

| 0nM Cort | 100nM Cort | Fold Change | Diff Score | Accession | Gene Symbol |
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| 197.2 | 64.1 | -3.1 | -25.716 | XM_192925.3 | Itch |
| 562 | 377.7 | -1.5 | -19.049 | NM_013565.2 | Itga3 |
| 524.4 | 221.3 | -2.4 | -42.689 | NM_010578.1 | Itgb1 |
| 271.3 | 158.8 | -1.7 | -19.247 | NM_010591.1 | Jun |
| 799.3 | 520.9 | -1.5 | -29.612 | NM_030887.2 | Jundm2 |
| 259.6 | 146.7 | -1.8 | -26.77 | NM_010593.1 | Jup |
| 315.8 | 150.7 | -2.1 | -66.866 | NM_008433.2 | Kcnn4 |
| 742.4 | 460.5 | -1.6 | -41.966 | NM_008443.2 | Kif3a |
| 192.2 | 98.3 | -2.0 | -28.944 | NM_010637 | Klf4 |
| 909.6 | 512.9 | -1.8 | -27.254 | NM_010637.1 | Klf4 |
| 421.3 | 250.5 | -1.7 | -30.696 | NM_010637.1 | Klf4 |
| 892.4 | 527.5 | -1.7 | -32.052 | NM_033563.2 | Klf7 |
| 374.4 | 252 | -1.5 | -25.263 | AK086122 | Klf7 |
| 352.5 | 235.5 | -1.5 | -22.634 | NM_153513.1 | L259 |
| 267.5 | 2198.9 | 8.2 | 18.551 | NM_008491.1 | Lcn2 |
| 1699.9 | 1138.8 | -1.5 | -42.401 | NM_172589.1 | Lhfpl2 |
| 3438.3 | 2099.1 | -1.6 | -24.587 | NM_019390.1 | Lmna |
| 7165.7 | 4937.3 | -1.5 | -17.844 | NM_019390.1 | Lmna |
| 7915.4 | 5402.8 | -1.5 | -19.482 | NM_010723.2 | Lmo4 |
| 126.8 | 67 | -1.9 | -22.155 | XM_149324.1 | LOC192195 |
| 129.3 | 50.9 | -2.5 | -37.162 | NM_199146.1 | LOC209387 |
| 435.1 | 231.5 | -1.9 | -58.563 | XM_135569.2 | LOC213480 |
| 472.9 | 320.5 | -1.5 | -17.281 | XM_128064.4 | LOC223672 |
| 148.9 | 91.5 | -1.6 | -18.863 | XM_123193.2 | LOC225021 |
| 804.7 | 380.3 | -2.1 | -21.922 | XM_135421.2 | LOC235973 |
| 749.6 | 514 | -1.5 | -27.742 | XM_142547.2 | LOC235979 |
| 7023.2 | 4426.9 | -1.6 | -58.762 | XM_143255.3 | LOC242051 |
| 156.7 | 54.3 | -2.9 | -22.439 | XM_193524.2 | LOC270589 |
| 4142.1 | 2740.5 | -1.5 | -17.924 | XM_287286.2 | LOC329750 |
| 1167.2 | 774 | -1.5 | -24.451 | XM_284733.2 | LOC331507 |
| 477.7 | 315.9 | -1.5 | -22.405 | XM_355056.1 | LOC381140 |
| 1001.6 | 674.9 | -1.5 | -30.44 | XM_358649.1 | LOC381739 |
| 308.5 | 207.3 | -1.5 | -22.173 | XM_355737.1 | LOC381747 |
| 489.2 | 173.3 | -2.8 | -51.318 | XM_356225.1 | LOC382128 |
| 977.4 | 488.5 | -2.0 | -67.574 | XM_356256.1 | LOC382157 |
| 138.7 | 64.6 | -2.1 | -21.127 | XM_359014.1 | LOC385959 |
| 444.4 | 210.2 | -2.1 | -25.885 | XM_359080.1 | LOC386124 |
| 447.1 | 287 | -1.6 | -20.936 | XM_359173.1 | LOC386330 |
| 228.4 | 136.9 | -1.7 | -26.926 | XM_359281.1 | Lst1 |
| 205.3 | 124.3 | -1.7 | -22.964 | NM_033509.2 | Ltap |
| 1499.2 | 3228.9 | 2.2 | 38.505 | NM_010741 | Ly6c |
| 124.7 | 297.5 | 2.4 | 19.745 | NM_016693 | Map3k6 |
| 858.6 | 492 | -1.7 | -66.94 | NM_138667.1 | Map3k7ip2 |
| 101 | 30.3 | -3.3 | -28.122 | NM_020007.2 | Mbnl1 |
| 967.8 | 342.2 | -2.8 | -33.63 | NM_008568.1 | Mcm7 |
| 293.6 | 163.2 | -1.8 | -44.924 | NM_010789.1 | Meis1 |
| 31.6 | 188.7 | 6.0 | 18.931 | NM_008581.1 | Mela |
| 69 | 162.9 | 2.4 | 23.33 | NM_008597.2 | Mglap |
| 2155.9 | 1479 | -1.5 | -27.128 | NM_008598.1 | Mgmt |

| 0nM Cort | 100nM Cort | Fold Change | Diff Score | Accession | Gene Symbol |
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| 990.6 | 635.5 | -1.6 | -25.319 | NM_025569.1 | Mgst3 |
| 1758.8 | 1190 | -1.5 | -18.365 | NM_025569.1 | Mgst3 |
| 201.6 | 379.5 | 1.9 | 20.138 | AK085856 | Mid2 |
| 2362 | 1618 | -1.5 | -39.796 | NM_021565.1 | Midn |
| 1152.7 | 1805.7 | 1.6 | 18.385 | NM_021462.2 | Mknk2 |
| 422.6 | 250.3 | -1.7 | -46.904 | NM_010807.2 | Mlp |
| 3962.5 | 2652.7 | -1.5 | -27.577 | NM_027098.1 | Mrpl30 |
| 509.2 | 275.2 | -1.9 | -58.275 | AK013126 | Mrpl38 |
| 919.9 | 248.1 | -3.7 | -38.588 | NM_011954.2 | Mrpplf3 |
| 2586.7 | 1683.4 | -1.5 | -24.237 | NM_080456.1 | Mrps6 |
| 23672 | 35348.5 | 1.5 | 17.333 | NM_013602.2 | Mt1 |
| 1166.1 | 4057.3 | 3.5 | 53.705 | NM_008630.1 | Mt2 |
| 681.8 | 295.4 | -2.3 | -80.752 | NM_008634.1 | Mtap1b |
| 1152.9 | 668.3 | -1.7 | -49.4 | NM_013827.1 | Mtf2 |
| 176.9 | 58.1 | -3.0 | -33.219 |  | mt-Nd4l |
| 190.2 | 101.2 | -1.9 | -25.145 | NM_181390.1 | Mustn1 |
| 576.9 | 843.1 | 1.5 | 30.5 | NM_177390 | Myo1d |
| 439.6 | 772.9 | 1.8 | 40.814 | NM_010875 | Ncam1 |
| 971.1 | 645.6 | -1.5 | -36.346 | NM_026554.2 | Ncbp2 |
| 1119.7 | 733 | -1.5 | -31.286 | NM_010880.2 | Ncl |
| 2374 | 1505.8 | -1.6 | -43.936 | NM_025316.2 | Ndufb5 |
| 283.9 | 158.8 | -1.8 | -20.328 | NM_010901.1 | Nfatc3 |
| 703.2 | 201.8 | -3.5 | -139.96 | NM_008688.2 | Nfic |
| 172.8 | 64 | -2.7 | -56.574 | NM_013609.1 | Ngfb |
| 3298.2 | 1962.8 | -1.7 | -28.666 | NM_018868.1 | Nol5 |
| 239.6 | 57.9 | -4.1 | -120.13 | NM_145434.1 | Nr1d1 |
| 759 | 510.2 | -1.5 | -24.015 | NM_008737.1 | Nrp |
| 203.6 | 118.3 | -1.7 | -25.196 | NM_008750 | Nxn |
| 1470.1 | 957.2 | -1.5 | -29.418 | NM_008750.2 | Nxn |
| 731 | 1363.7 | 1.9 | 59.529 | NM_053109.1 | Ocil |
| 334.2 | 180.5 | -1.9 | -21.37 | NM_133859.1 | Olfml3 |
| 880.3 | 511.2 | -1.7 | -56.375 | NM_027881.1 | Osbpl3 |
| 626.9 | 377.8 | -1.7 | -37.989 | NM_011119.1 | Pa2g4 |
| 2135.4 | 1353.4 | -1.6 | -28.218 | NM_008776.1 | Pafah1b3 |
| 309.5 | 212.2 | -1.5 | -17.97 | NM_026420.1 | Paip2 |
| 308.9 | 172.5 | -1.8 | -42.796 | NM_011112.1 | Papola |
| 246.3 | 132.6 | -1.9 | -32.355 | NM_008793.1 | Pcsk4 |
| 125.7 | 73.3 | -1.7 | -18.085 | NM_009981.2 | Pcyt1a |
| 3351.5 | 1977 | -1.7 | -61.336 | XM_132501.2 | Pdap1 |
| 188.2 | 60.3 | -3.1 | -32.109 | NM_012019.2 | Pdcd8 |
| 635.9 | 350.7 | -1.8 | -28.921 | NM_008808.2 | Pdgfa |
| 1023.5 | 626.1 | -1.6 | -36.813 | NM_008808 | Pdgfa |
| 316.4 | 1030.6 | 3.3 | 27.946 | NM_013743.1 | Pdk4 |
| 52 | 290 | 5.6 | 122.373 | NM_011065.2 | Per1 |
| 198.9 | 51.9 | -3.8 | -93.06 | NM_011066.1 | Per2 |
| 176.6 | 49.1 | -3.6 | -75.06 | NM_011067 | Per3 |
| 245.5 | 356.2 | 1.5 | 22.633 | NM_172783.1 | Phka2 |
| 957.7 | 321.6 | -3.0 | -65.461 | NM_009344.1 | Phlda1 |
| 542.4 | 1077.2 | 2.0 | 48.417 | NM_011085 | Pik3r1 |

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| 206.7 | 128.7 | -1.6 | -24.168 | NM_181585.3 | Pik3r3 |
| 793.8 | 501.5 | -1.6 | -41.926 | XM_136134.3 | Pir |
| 1244.2 | 808.8 | -1.5 | -39.909 | NM_144859.1 | Pja2 |
| 384.1 | 249.3 | -1.5 | -22.756 | NM_018807.3 | Plagl2 |
| 187.9 | 70.8 | -2.7 | -39.862 | NM_011113.2 | Plaur |
| 421.3 | 174.2 | -2.4 | -82.029 | NM_011117 | Plec1 |
| 607.7 | 146.4 | -4.2 | -104.48 | NM_031191.1 | Plf |
| 3858.1 | 1002.3 | -3.8 | -100.80 | NM_031191 | Plf |
| 1330 | 410.4 | -3.2 | -38.405 | NM_011118.1 | Plf2 |
| 2457.4 | 1391.9 | -1.8 | -57.192 | NM_152804.1 | Plk2 |
| 652 | 443.8 | -1.5 | -30.944 | NM_148932.1 | Pom121 |
| 530.2 | 215.4 | -2.5 | -121.52 | NM_080555.1 | Ppap2b |
| 141.8 | 69.6 | -2.0 | -29.274 | NM_175934.2 | Ppp1r10 |
| 3430.8 | 2064.8 | -1.7 | -30.714 | NM_008913.1 | Ppp3ca |
| 476.7 | 1443 | 3.0 | 31.507 | NM_054077.2 | Prelp |
| 2679.8 | 1685.5 | -1.6 | -42.699 | NM_011100.3 | Prkacb |
| 308.2 | 194.8 | -1.6 | -30.48 | NM_011158.2 | Prkar2b |
| 214.4 | 122.2 | -1.8 | -28.746 | NM_011101.1 | Prkca |
| 381.4 | 233.4 | -1.6 | -20.478 | NM_022999.1 | Prrg2 |
| 281.8 | 137.9 | -2.0 | -26.138 | NM_011127.1 | Prrx1 |
| 713.7 | 391.9 | -1.8 | -40.57 | NM_133948.2 | Psip1 |
| 2373 | 1382.9 | -1.7 | -33.792 | NM_011191.1 | Psme2b |
| 208.4 | 107.2 | -1.9 | -40.531 | NM_008969.1 | Ptgs1 |
| 2104.3 | 1215.9 | -1.7 | -53.061 | NM_008973.1 | Ptn |
| 275.7 | 490.8 | 1.8 | 19.658 | NM_023587 | Ptplb |
| 267.9 | 102.4 | -2.6 | -84.577 | NM_011202.2 | Ptpn11 |
| 396.4 | 240.8 | -1.6 | -21.375 | NM_011221.1 | Purb |
| 115.8 | 59.1 | -2.0 | -18.042 | NM_009021.1 | Rai1 |
| 135.1 | 71 | -1.9 | -23.887 | NM_028712.3 | Rap2b |
| 258.8 | 474.9 | 1.8 | 52.396 | NM_009025.1 | Rasa3 |
| 439.7 | 240.3 | -1.8 | -35.045 | NM_148930.2 | Rbm5 |
| 2261 | 1545.5 | -1.5 | -29.797 | NM_011254.2 | Rbp1 |
| 412.8 | 247.1 | -1.7 | -39.169 | NM_054048.1 | Rcor1 |
| 342.5 | 205.8 | -1.7 | -39.393 | NM_009041 | Rdx |
| 785 | 1249.9 | 1.6 | 20.751 | NM_133955.1 | Rhou |
| 187 | 275.3 | 1.5 | 20.837 | NM_177620.2 | Rin3 |
| 347.6 | 176.8 | -2.0 | -62.402 | NM_133242.1 | Rnpc2 |
| 649.8 | 447.8 | -1.5 | -19.061 | NM_145620.3 | Rnu3ip2 |
| 393.2 | 223.4 | -1.8 | -36.044 | NM_018739.1 | Rp9h |
| 1705.5 | 921.3 | -1.9 | -60.181 | NM_009083.2 | Rpl30 |
| 247.4 | 132.2 | -1.9 | -39.043 | NM_009088 | Rpo1-4 |
| 127.2 | 58.5 | -2.2 | -19.258 | NM_009821.1 | Runx1 |
| 364.7 | 176.8 | -2.1 | -31.946 | NM_011310.1 | S100a3 |
| 86.1 | 156.4 | 1.8 | 20.62 | NM_011315 | Saa3 |
| 2156.4 | 1429 | -1.5 | -35.826 | NM_009125.1 | Sca2 |
| 912.8 | 1395.7 | 1.5 | 36.973 | NM_138741.1 | Sdpr |
| 106 | 49.3 | -2.2 | -17.77 | NM_009153.1 | Sema3b |
| 276.7 | 184.4 | -1.5 | -19.086 | NM_144851.2 | Senp1 |
| 591.5 | 190.5 | -3.1 | -86.935 | NM_198247.1 | Sertad4 |

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| 227.2 | 435.5 | 1.9 | 55.793 | XM_125538.4 | Sesn1 |
| 1481 | 2488.6 | 1.7 | 45.63 | NM_011361.1 | Sgk |
| 402.9 | 192.5 | -2.1 | -49.134 | NM_013665.1 | Shox2 |
| 241.1 | 153.5 | -1.6 | -26.116 | NM_009177.2 | Siat4a |
| 1543.7 | 695.1 | -2.2 | -54.989 | NM_013612.1 | Slc11a1 |
| 267.7 | 154.4 | -1.7 | -34.94 | XM_147798.4 | Slc4a7 |
| 895.1 | 566.8 | -1.6 | -20.179 | NM_016769 | Smad3 |
| 135.9 | 80.2 | -1.7 | -19.096 | AK028739 | Smcx |
| 69.9 | 127.7 | 1.8 | 20.025 | NM_021491.2 | Smpd3 |
| 1613.8 | 864.1 | -1.9 | -23.931 | NM_009214 | Sms |
| 62.8 | 120.6 | 1.9 | 18.398 | NM_010831.1 | Snf1lk |
| 1348.4 | 529.6 | -2.5 | -73.252 | NM_019654.2 | Socs5 |
| 102.1 | 487.8 | 4.8 | 112.369 | NM_011451.1 | Sphk1 |
| 307.4 | 169.9 | -1.8 | -41.112 | NM_133903.2 | Spon2 |
| 1368.5 | 756.4 | -1.8 | -22.468 | NM_009263.1 | Spp1 |
| 275.2 | 165 | -1.7 | -34.794 | AK080894 | Srb1 |
| 253.1 | 161.3 | -1.6 | -21.587 | NM_025527.1 | Srp19 |
| 8653.2 | 5828.5 | -1.5 | -32.211 | AK080830 | Srr |
| 556.5 | 375 | -1.5 | -33.279 | AK043819 | Srr |
| 134.5 | 43.9 | -3.1 | -47.902 | NM_147155 | Tagap |
| 100.9 | 26.5 | -3.8 | -24.184 | NM_019512.1 | Tcerg1 |
| 3038.2 | 1955 | -1.6 | -29.806 | NM_011552.1 | Tcof1 |
| 203.3 | 327.7 | 1.6 | 19.032 | NM_011567.1 | Tead4 |
| 1549 | 463.2 | -3.3 | -194.87 | NM_017376.2 | Tef |
| 170.7 | 96.7 | -1.8 | -26.824 | XM_109868 | Tens1 |
| 1305.8 | 736.9 | -1.8 | -76.024 | NM_009371.2 | Tgfbr2 |
| 398.8 | 723 | 1.8 | 55.251 | NM_011578 | Tgfbr3 |
| 774.8 | 502.3 | -1.5 | -21.495 | NM_009372.2 | Tgif |
| 3436.4 | 2145.6 | -1.6 | -31.818 | NM_011593 | Timp1 |
| 2251.1 | 1484 | -1.5 | -32.819 | NM_011595.1 | Timp3 |
| 272.3 | 170.8 | -1.6 | -29.995 | NM_144792.2 | Tmem23 |
| 3383.6 | 2048.8 | -1.7 | -50.187 | NM_013749.1 | Tnfrsf12a |
| 110.3 | 45.5 | -2.4 | -28.592 | NM_009393.1 | Tnnc1 |
| 280.5 | 190.5 | -1.5 | -17.469 | NM_133673.2 | Tor1b |
| 1011.1 | 633.1 | -1.6 | -22.532 | NM_145853.2 | Tpcn1 |
| 700.4 | 456.7 | -1.5 | -28.907 | NM_024427 | Tpm1 |
| 1424.4 | 699.5 | -2.0 | -32.809 | NM_022314 | Tpm3 |
| 102 | 53.7 | -1.9 | -17.735 | NM_144549 | Trib1 |
| 264.6 | 126.2 | -2.1 | -52.584 | NM_201373.1 | Trim56 |
| 3939.9 | 2642.1 | -1.5 | -23.623 | NM_053100.1 | Trim8 |
| 290.1 | 164.1 | -1.8 | -21.19 | NM_011653 | Tuba1 |
| 293 | 150.2 | -2.0 | -30.247 | NM_008807.1 | Tulp2 |
| 1708.1 | 1138.6 | -1.5 | -43.616 | NM_007855.1 | Twist2 |
| 547.9 | 311.5 | -1.8 | -26.208 | NM_172253.1 | Twistnb |
| 442.6 | 254.5 | -1.7 | -39.948 | NM_172253.1 | Twistnb |
| 275.7 | 149.1 | -1.8 | -31.803 | NM_029770.1 | Unc5b |
| 349.9 | 185.3 | -1.9 | -24.047 | NM_146144.2 | Usp1 |

| 0nM Cort | 100nM Cort | Fold Change | Diff Score | Accession | Gene Symbol |
|---|---|---|---|---|---|
| 288.4 | 184.8 | -1.6 | -29.02 | NM_009462.1 | Usp10 |
| 290.2 | 145.8 | -2.0 | -51.708 | NM_176972 | Usp37 |
| 360.5 | 153.8 | -2.3 | -29.705 | XM_135857.4 | Utp14a |
| 2375.1 | 1406.7 | -1.7 | -42.628 | NM_011693.2 | Vcam1 |
| 1094 | 688.7 | -1.6 | -28.392 | NM_009505.2 | Vegfa |
| 141.7 | 72.8 | -1.9 | -25.811 | NM_027462.2 | Wars2 |
| 1099.3 | 705.3 | -1.6 | -42.304 | NM_031877.2 | Wasf1 |
| 1160.3 | 676.1 | -1.7 | -24.362 | NM_009517.1 | Wig1 |
| 1794.8 | 1020 | -1.8 | -39.86 | NM_018865.1 | Wisp1 |
| 123.3 | 66.1 | -1.9 | -21.061 | AK049134 | XEDAR EDA-A2R |
| 131.9 | 71.6 | -1.8 | -21.956 | NM_178074.2 | Xpnpep2 |
| 16931 | 10123.3 | -1.7 | -36.19 | AK029441 | Ybx3 |
| 472.5 | 137.8 | -3.4 | -47.969 | NM_010731.1 | Zbtb7 |
| 229.6 | 129.8 | -1.8 | -22.591 | NM_153538.1 | Zcchc6 |
| 180.3 | 85.5 | -2.1 | -21.181 | NM_027494.1 | Zcchc8 |
| 281.8 | 115.5 | -2.4 | -69.495 | NM_018759.1 | Zfp326 |
| 205.3 | 137.8 | -1.5 | -18.039 | NM_011759.1 | Zfp41 |
| 103.2 | 52.5 | -2.0 | -19.081 | NM_173364 | Zfp445 |
| 317.8 | 78.7 | -4.0 | -60.684 | NM_198416.1 | Zzz3 |
| 2981.1 | 1144.2 | -2.6 | -72.14 | | |
| 895.7 | 388 | -2.3 | -84.092 | AK081844.1 | |
| 1963.9 | 990.9 | -2.0 | -24.852 | AK002910.1 | |
| 11284.2 | 7170.2 | -1.6 | -21.622 | BC029319.1 | |
| 223.6 | 147.6 | -1.5 | -21.798 | AK053042.1 | |
| 243.2 | 150.7 | -1.6 | -21.158 | | 0610007N19Rik |
| 402.3 | 191.7 | -2.1 | -78.588 | NM_025793.1 | 0610008N23Rik |
| 311 | 183 | -1.7 | -20.746 | NM_025339.2 | 0610027O18Rik |
| 263.1 | 138 | -1.9 | -25.843 | NM_172401.2 | 1110057K04Rik |
| 888.1 | 569.8 | -1.6 | -43.593 | XM_147036.4 | 1190002N15Rik |
| 600.7 | 314.1 | -1.9 | -49.456 | NM_025822.2 | 1200013F24Rik |
| 324.7 | 725.6 | 2.2 | 18.248 | NM_133753.1 | 1300002F13Rik |
| 790.1 | 326.7 | -2.4 | -70.78 | XM_132343.3 | 1500011J06Rik |
| 228 | 121.9 | -1.9 | -17.63 | XM_356257.1 | 1500041I23Rik |

Listed from left to right, the average signal intensity of each gene in the vehicle treated control (0nM Cort), 100nM corticosterone treated group (100nM Cort), the fold change in expression level from basal to corticosterone treatment, the significance value in Diff. score (Diff. 17 is equivalent to p=0.02, larger Diff. scores are lower p-values), the accession number, and the gene symbol for each gene regulated in HT-22/MR16 cells.

A.

QuickTime™ and a
TIFF (Uncompressed) decompressor
are needed to see this picture.

B.

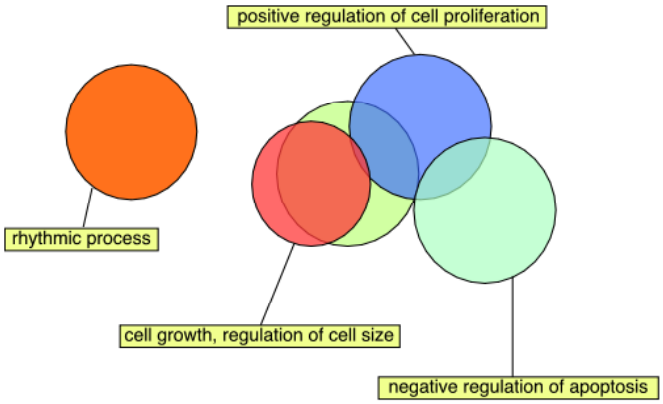



**Figure 4-4 Gene Ontology of genes regulated by 100nm corticosterone in HT-22 MR16 cells.**

A) Quantitation of genes that sort into different functional categories under the ontology of Biological Process as determined by GOTM software. Categories in red denote an overrepresentation of regulated genes relative to the expected number of genes within that category. B) Vennmaster diagram depicting overlapping functional categories of genes that were also determined to be overrepresented in the data as determined by GoMiner and VennMaster software.

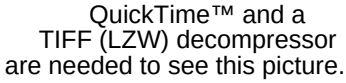



**Figure 4-5 Venn diagram representing the overlap between cell treatment groups of genes that are regulated by corticosterone.**

P0-P100=HT-22 Parent 100nM corticosterone, M0-M1=HT-22 MR16 1nM cortiocsterone, and M0-M100=HT-22 MR16 100nM corticosterone

**Table 4-5 Corticosterone-responsive genes per group from venn diagram in Figure 5.**

| P0-P100 (192) | | | M0-M100 (408) | | | | | M0-M1 (7) | P100 & M100 (37) | M1 & M100 (11) | M1 & P100 (2) | P100 & M100 & M1 (21) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AL033326 | Hspd1 | Snrp70 | Acadm | E2f3 | Lhfpl2 | Rap2b | Wasf1 | C3 | Chrd | 5730594E01Rik | Nr1h4 | Arntl |
| Abcf2 | Idb2 | Snrpa | Aebp1 | EDA-A2R | Lmna | Rasa3 | Wig1 | C330004K0ik1R | D15Mit260 | B230387C07Rik | P2rx5 | Chst1 |
| Acas2 | Idh3g | Srm | Akap9 | Eda2r | Lmo4 | Rbm5 | XEDAR | Cxcl1 | Dnmt3l | Fbxw7 | | Col8a1 |
| Akr1c12 | Il1rl1 | Suclg1 | Akt3 | Edg2 | Lst1 | Rbp1 | Xpnpep2 | Dpysl3 | Dusp1 | Lcn2 | | Cp |
| Anapc5 | Irak1 | Taf7l | Aldh2 | Efemp2 | Ltap | Rcor1 | Ybx3 | Isg20 | Dusp4 | Mela | | Cspg2 |
| Ankrd1 | Itgb7 | Tcp1 | Ampd3 | Eif5b | Map3k7ip2 | Rdx | Zbtb7 | Vdr | Dusp8 | Nfic | | Cyr61 |
| Anxa4 | Khdrbs1 | Tes | Angptl2 | Ela1 | Mbnl1 | Rhou | Zcchc6 | Wisp2 | Epha2 | Pdcd8 | | D15Bwg0759e |
| Aptx | Lynx1 | Tm4sf8 | Angptl4 | Elf2 | Mcm7 | Rnpc2 | Zcchc8 | | Esd | Per2 | | Dbp |
| Aqp5 | Man2a1 | Tm7sf1 | Ankhd1 | Emilin1 | Meis1 | Rnu3ip2 | Zfp326 | | Hs6st1 | Plaur | | Fkbp5 |
| Arf5 | Mapk6 | Tnfrsf22 | Aqp1 | Eml1 | Mgmt | Rp9h | Zfp41 | | Jun | Socs5 | | Mt2 |
| Arpc1a | Mat2a | Tnfrsf6 | Arhgap22 | Eml4 | Mgst3 | Rpl30 | Zfp445 | | Klf4 | Spp1 | | Ncam1 |
| Asns | Mbd3 | Tnk2 | Arid5a | Emp1 | Mid2 | Rpo1-4 | Zzz3 | | LOC329750 | | | Nr1d1 |
| Atf4 | Mbtps1 | Top2a | Ase1 | Emp3 | Midn | Runx1 | | | Ly6c | | | Pdk4 |
| Atic | Mrpl3 | Tpm4 | Ash1l | Enah | Mlp | S100a3 | | | Map3k6 | | | Per1 |
| Atp6ap2 | Mrpl48 | Trib3 | Atbf1 | Enpp4 | Mrpl30 | Sca2 | | | Mglap | | | Per3 |
| Aup1 | Msln | Tsrc1 | Atp5e | Ephb2 | Mrpl38 | Sdpr | | | Mknk2 | | | Phlda1 |
| Bnip1 | Msn | Tubb5 | Atp5k | Etv4 | Mrps6 | Sema3b | | | Mrpplf3 | | | Plf |
| Brd7 | Mtch1 | Ube2e3 | Bcl10 | Exosc1 | Mt1 | Senp1 | | | Ncbp2 | | | Prelp |
| Btg1 | Mybbp1a | Uble1a | Bcl6 | Ezh2 | Mtap1b | Sertad4 | | | Ngfb | | | Sgk |
| C1qbp | Myc | Uchl5 | Bhlhb2 | Fbln2 | Mtf2 | Shox2 | | | Pa2g4 | | | Sphk1 |
| Camk1 | Napa | Umps | Brd4 | Fibp | Mustn1 | Siat4a | | | Plec1 | | | Tef |
| Cdh2 | Nfkb1 | Vav2 | Brunol4 | Fmnl2 | Myo1d | Slc11a1 | | | Plf2 | | | |
| Cdk4 | Nipsnap3b | Xab1 | Capn2 | Fosl1 | Ncl | Slc4a7 | | | Plk2 | | | |
| Cryab | Nnmt | Xpot | Casp3 | Gadd45b | Ndufb5 | Smad3 | | | Prkar2b | | | |
| Cryz | Nol5a | Yeats4 | Ccnd1 | Galnt1 | Nfatc3 | Smcx | | | Rin3 | | | |
| Csda | Nradd | Ywhaq | Ccnd2 | Gbf1 | Nol5 | Sms | | | Saa3 | | | |
| Ctps | Nubp2 | Zcchc14 | Ccng1 | Gbif | Nrp | Snf1lk | | | Sesn1 | | | |
| Dhps | Obfc1 | Zfp330 | Cd164l1 | Gkap1 | Nxn | Spon2 | | | Smpd3 | | | |
| Dysf | Odz4 | Zranb1 | Cd34 | Glipr1 | Ocil | Srb1 | | | Tead4 | | | |
| Eef2 | Pigt | | Cd44 | Glrx2 | Olfml3 | Srp19 | | | Timp3 | | | |
| Eif2b1 | Plekhj1 | | Cdc25a | Gng8 | Osbpl3 | Srr | | | Tnfrsf12a | | | |
| Eif3s8 | Ppp1ca | | Cdc2l2 | Gnpda2 | Pafah1b3 | Tagap | | | Wisp1 | | | |
| Eif3s9 | Ppp2r1a | | Cdkn1a | Golga2 | Paip2 | Tcerg1 | | | | | | |
| Eif4a2 | Prnpip1 | | Chd1 | Golph4 | Papola | Tcof1 | | | | | | |

Table 5. Continued

| P0-P100 (192) | | M0-M100 (408) | | | | M0-M1 (7) | P100 & M100 (37) | M1 & M100 (11) | M1 & P100 (2) | P100 & M100 & M1 (21) |
|---|---|---|---|---|---|---|---|---|---|---|
| Eif5a | Prss11 | Chd4 | Gpc1 | Pcsk4 | Tens1 | | | | | |
| Eps8 | Prss15 | Clspn | Gpr149 | Pcyt1a | Tgfbr2 | | | | | |
| Etfa | Psma1 | Cnn2 | Grb10 | Pdap1 | Tgfbr3 | | | | | |
| Fancl | Psmd13 | Cnot4 | Gsn | Pdgfa | Tgif | | | | | |
| Fdps | Psmd7 | Cntf | H1f0 | Phka2 | Timp1 | | | | | |
| Figf | Psme2 | Col1a2 | H2-M3 | Pik3r1 | Tmem23 | | | | | |
| Flnb | Ptp4a2 | Copeb | Hcapg-pending | Pik3r3 | Tnnc1 | | | | | |
| Fn1 | Rad23a | Cox7b | Hirip5 | Pir | Tor1b | | | | | |
| Fosl2 | Ranbp9 | Crebbp | Hist1h2ae | Pja2 | Tpcn1 | | | | | |
| Fxr2h | Rbbp7 | Cryzl1 | Hist1h2ao | Plagl2 | Tpm1 | | | | | |
| G3bp | Rbp2 | Csf1 | Hoxc6 | Pom121 | Tpm3 | | | | | |
| G6pc3 | Rhoj | Csnk2a1-rs3 | Hrb | Ppap2b | Trib1 | | | | | |
| Gadd45a | Riok1 | Cstf3 | Hspa5bp1 | Ppp1r10 | Trim56 | | | | | |
| Glrx1 | Rtn4 | Cugbp1 | Hspg2 | Ppp3ca | Trim8 | | | | | |
| Gna11 | Ruvbl1 | Ddah2 | Ide | Prkacb | Tuba1 | | | | | |
| Gnas | Rwdd1 | Ddef1 | Igfbp4 | Prkca | Tulp2 | | | | | |
| Gpiap1 | S3-12 | Ddx21 | Igfbp6 | Prrg2 | Twist2 | | | | | |
| Grwd1 | Sdbcag84 | Ddx24 | Iqgap1 | Prrx1 | Twistnb | | | | | |
| Gtf3a | Sfrs10 | Ddx6 | Itch | Psip1 | Unc5b | | | | | |
| Herpud1 | Sfrs5 | Dek | Itga3 | Psme2b | Usp1 | | | | | |
| Hnrpa2b1 | Sh3bgrl2 | Dfna5h | Itgb1 | Ptgs1 | Usp10 | | | | | |
| Hnrpk | Sh3bp2 | Diap3 | Jundm2 | Ptn | Usp37 | | | | | |
| Hoxd8 | Sh3kbp1 | Dok1 | Jup | Ptplb | Utp14a | | | | | |
| Hrbl | Smarce1 | Dpm3 | Kcnn4 | Ptpn11 | Vcam1 | | | | | |
| Hspca | Smc1l1 | Dsip1 | Kif3a | Purb | Vegfa | | | | | |
| Hspcb | Smu1 | Dub1 | Klf7 | Rai1 | Wars2 | | | | | |

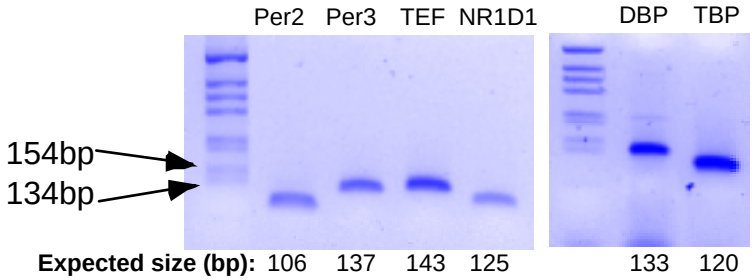
regulated genes in the P0-P100 group, 192 (76%) of them were exclusive to this group, suggesting that they may be genes that are regulated exclusively by GR. In the M0-M100 category, 86% (407 of 476) of the corticosterone-regulated genes were specific for this group. In the M0-M1 category, only 7 of the 41 genes (17%) were specific for this group. The most striking feature of this figure is the small degree of overlap between the P0-P100 and the M0-M100 groups- only 58 genes (23% of the P0-P100 group and 12% of the M0-M100 group) overlapped between them, suggesting distinct sets of GR and MR/GR responsive genes.

*Quantitative RT-PCR (Q-RT-PCR) confirmation of microarray results:*

Q-RT-PCR was performed on 5 genes in the circadian rhythm category of genes that was identified as having been significantly overrepresented in all three treatment groups. This category contained genes that were common to the P0-P100, M0-M1, and M0-M100 groups, and one gene, Per2, that was only regulated in the M0-M1 and M0-M100 groups. None of the genes were regulated in the P0-P1 treatment group. To first confirm that the genes of interest are expressed in HT-22 cells, RT-PCR was performed with cDNA from HT-22 cells using primer pairs specific to each gene and the amplified products were separated on 2% agarose gels along with a DNA ladder to demonstrate that the amplification products were the expected sizes (Figure 4-6A).

To determine whether corticosterone regulates the expression of these genes in a similar manner to that observed in the microarray, cDNA was made from the same stocks of RNA that were utilized for the microarray experiment and Q-RT-PCR was performed on vehicle treated and corticosterone treated samples with the same primer pair used in

A.




B.

| HT22 MR16: Fold Change | | | HT22 Parent: Fold Change | | |
|---|---|---|---|---|---|
| **Gene** | **qRT-PCR** | **Microarray** | **Gene** | **qRT-PCR** | **Microarray** |
| **Per2** | | | **Per2** | | |
| 1nM | -1.2 | -1.6 | 1nM | -1.2 | NC |
| 100nM | -2.2 | -3.8 | 100nM | -1.3 | NC |
| **Per3** | | | **Per3** | | |
| 1nM | -1.6 | -2 | 1nM | -1.1 | NC |
| 100nM | -3.8 | -3.6 | 100nM | -2.6 | -2.3 |
| **Dbp** | | | **Dbp** | | |
| 1nM | -2.3 | -2.8 | 1nM | -1.1 | NC |
| 100nM | -7.3 | -16.1 | 100nM | -4.9 | -9.8 |
| **TEF** | | | **TEF** | | |
| 1nM | -1.4 | -1.9 | 1nM | -1.2 | NC |
| 100nM | -3.1 | -3.3 | 100nM | -3.9 | -2.8 |
| **NR1D1** | | | **NR1D1** | | |
| 1nM | -1.4 | -1.9 | 1nM | NC | NC |
| 100nM | -1.4 | -4.1 | 100nM | -1.2 | -2.6 |

**Figure 4-6 Quantitative RT-PCR confirmations of microarray data**

A) Amplified fragments of gene specific cDNAs from HT-22 cells. cDNA was generated from unstimulated HT-22 cells and PCR was performed with primer pairs specific for each gene listed. The expected amplicon sizes are depicted below each lane. B) Q-RT-PCR confirmations of corticosterone responsive genes from microarray.

the test RT-PCR. Figure 4-6B depicts the Q-RT-PCR data from the 1nM and 100nM treated HT-22 Parent and HT-22 MR16 cells as fold change over vehicle treated cells. The corresponding fold change in expression of the same gene from the microarray data is shown for comparison. Comparison of the Q-RT-PCR data with the microarray data for all of the genes analyzed reveals fairly consistent results. Overall, the Q-RT-PCR fold change values were slightly less than the corresponding microarray fold change values, but the same pattern of regulation was observed. None of the genes in the HT-22 Parent 1nM corticosterone treated group appeared to be regulated by corticosterone to any significant degree in either the microarray or Q-RT-PCR.

***Discussion:***

Using the Illumina Whole Genome Expression Arrays (BeadChips), we have identified 677 corticosterone-responsive genes in HT-22 and HT-22/MR16 cells. In the HT-22 Parent cell line that expresses only GR, 252 genes were identified as corticosterone-responsive after incubation with 100nM corticosterone. In HT-22/MR16 cells that express both MR and GR, 41 genes were regulated by 1nM corticosterone while 476 genes were identified as regulated by 100nM corticosterone. Gene ontology analyses demonstrate the largest number of genes regulated by corticosterone in each group are involved in cellular metabolism, intracellular signaling cascades, and the regulation of transcription.

Several studies have suggested that the Illumina bead array platform is a very sensitive and reliable expression profiling technique (Liu, Shin et al. 2006). Consistent with this, we have identified many corticosteroid-responsive genes in our study in HT-22

cells whose expression was previously shown to be regulated by corticosterone in hippocampus. For example, the well known glucocorticoid target gene, serum and glucocorticoid regulated kinase (SGK1) was clearly upregulated in M1, M100, and P100 samples. Metallothionein 1 (Mt1) was up-regulated in HT-22/MR16 cells in response to 100nM corticosterone, and metallothionein 2 (Mt2) was up-regulated in M1, M100, and P100. These genes are regulated in the hippocampus *in vivo* through adrenalectomy and stress (Gasull, Giralt et al. 1994; Belloso, Hernandez et al. 1996). More generally, the effects of corticosterone on cellular energy metabolism have also been described (Plaschke, Muller et al. 1996), consistent with the pathway analysis of regulated genes in these cell lines. However, it should be noted that our study did not identify some genes that have been shown to be corticosteroid-responsive in hippocampus such as the serotonin receptor 1a (5HT1a). This suggests that the level of sensitivity of this microarray may still preclude the identification of low abundance transcripts that may in fact be regulated by GR and/or MR. A recent paper addressing the sensitivity of large-scale transcriptome monitoring across different platforms (Serial Analysis of Gene Expression [SAGE] and Affymetrix Gene Chips) found that a large percentage of low abundance genes are not reliably detectable (Evans, Datson et al. 2002). In the present study, the low abundance genes (average intensity values < 100) were not included in the analysis as high variability in expression between the triplicate samples precluded an efficient analysis of their patterns of gene expression.

Our experimental design allowed for the detection of genes that were regulated exclusively by GR in HT-22 cells and genes that were regulated predominately by MR or both MR and GR together in the HT-22/MR16 cells. Interestingly, however, only 58

genes overlapped between the HT-22 100nM (P100) and HT-22/MR16 (M100) groups (23% of P100, and 12% of M100), suggesting that when MR and GR are activated together, different sets of genes may be activated compared to those activated by GR alone. In HT-22/MR16 cells treated with 1nM corticosterone (predominant MR activation), 41 genes were differentially expressed from vehicle treated cells. Of these 41 genes, 7 (17%) were exclusive to the 1nM treated group, while 21 (51%) overlapped with both the M100 and P100 groups and 11 (27%) were found to overlap with the M100 group, suggesting that greater than 75% of MR responsive genes are also responsive to MR/GR together.

These results largely confirm and extend the studies by Datson et al. that identified corticosteroid-responsive genes in rat hippocampus using SAGE. They used ADX rats that had been replaced with low or high levels of corticosterone to study MR-responsive and GR/MR-responsive genes. Similar to results here, they found that a large percentage of the corticosteroid-reponsive genes were involved in cellular processes such as metabolism, energy expenditure, protein synthesis and signaling. Similar to our results, they also found that approximately 50% of MR-responsive genes were negatively regulated by corticosteroid, while significantly greater than 50% of the GR/MR-responsive genes were negatively regulated by corticosterone. However, it should be noted that their SAGE studies identified only 203 corticosterone-responsive genes, while our gene expression profiles identified 485 corticosterone-responsive genes in HT-22/MR16 cells (most similar to their experimental groups). In addition, our studies in the parent HT-22 cells identified an additional 192 corticosterone-regulated genes. Thus, the increased sensitivity of our studies and additional comparison with HT-22 cells (GR-

responsive genes) significantly increased the information on glucocorticoid-regulated gene expression profiles in hippocampus.

It was most interesting to note that one of the significant differences in the gene ontology profiles between GR-activated genes (P100) and MR/GR activated genes (M100) was negative regulation of apoptosis. Several studies have shown that high levels of corticosteroids can have deleterious effects on hippocampal neurons, greatly enhancing the likelihood that subsequent insults, such as increased levels of glutamate, will kill cells in region CA1. The molecular mechanisms underlying these processes are not completely understood, but evidence suggests that a balance in the expression and activation levels of MR and GR may be important. A predominance of GR activation may be responsible for many of the adverse effects of corticosterone on the hippocampus, while MR activation may provide more of a neuroprotective role (Herman and Seroogy 2006).

Of particular interest, two pro-apoptotic genes caspase-3 and calpain-2 are down-regulated exclusively in HT-22/MR16 cells treated with 100nM corticosterone. Additionally, metallothionein I, which was also exclusively up-regulated in HT-22/MR16 cells treated with 100nM corticosterone, has been shown to enhance cell survival following exposure to oxidative stress (Levadoux-Martin, Hesketh et al. 2001). These data suggest that when the expression patterns of large numbers of genes are changed by the concurrent activation of MR and GR together relative to GR activation alone, the expression patterns of categories of genes with potentially biologically significant effects relevant to hippocampal structure and function may also be altered.

In addition to interesting pathways of genes that were altered differentially

between these two cell lines, there was one pathway of genes in particular that was identified as being regulated by corticosterone in M1, M100, and P100. Of the 21 genes that were in common between these three groups, 6 (29%) of them were genes involved in circadian rhythmic processes including Per1, Per3, NR1D1 (REV-ERBα), Dbp, Tef, and Arntl (Bmal1). Furthermore, Per2 was also found to be regulated by 1nM and 100nM corticosterone, but only in HT-22 MR16. We have confirmed these changes in HT-22 cell lines using Q-RT-PCR. The altered expression of many of these circadian rhythm genes has been associated with several mood disorders, including seasonal affective disorder (SAD) (Partonen, Treutlein et al. 2007) and bipolar disorder (Mansour, Wood et al. 2006; Nievergelt, Kripke et al. 2006). Interestingly, some recent papers have suggested that neurogenesis in the hippocampus fluctuates in a circadian fashion and that progenitor cell proliferation in the dentate gyrus is inhibited by Per2 (Borges 2004; Guzman-Marin, Suntsova et al. 2007). These data suggest that the improper regulation of circadian control genes in the hippocampus by corticosterone may be important in many hippocampal functions including mood disorders.

Finally, however, it should be noted that within the HT-22/MR16 cells treated with 100nM corticosterone, it is not possible to distinguish between genes that are regulated by the activation of homodimers versus heterodimers. In addition, as all of these cells were treated with corticosterone for 24 hours, it is not possible to tell whether the regulation of any particular gene is due to a primary action of MR or GR as a transcription factor on the promoter, or a secondary or even tertiary regulation of a downstream effector. The analysis of gene expression in these cells using a time-course analysis starting as soon as 1 hour or less after the addition of corticosterone would be

appropriate for the identification of large numbers of genes that may be primarily regulated by direct MR/GR/promoter interactions.

In conclusion, using the Illumina Bead Array microarray system, we have identified 677 corticosterone-responsive genes in HT-22 Parent and HT-22/MR16 cells under different corticosterone concentrations. This system has allowed us to identify genes that are regulated by GR alone, MR alone, and MR and GR co-activation. Furthermore, this system has allowed us to determine the effects of an altered MR:GR ratio on global gene transcription output in response to the same dose of corticosterone. Gene ontologies that are overrepresented in the data suggest that corticosterone has significant effects on the expression patterns of genes involved in cellular metabolism and circadian rhythm. Gene ontologies that are overrepresented differentially between HT-22 Parent and HT-22/MR16 cells suggest that the co-activation of MR and GR versus GR activation alone results in altered expression patterns of a number of genes that are involved in the regulation of apoptosis and cell proliferation. Together, these results provide possible insights into both the similar and differential modes of action for GR and MR in mediating a wide variety of hippocampal functions, including neuronal survival and proliferation.

# Chapter 5

## Conclusions and future directions

### Summary of contributions:

### Introduction:

Glucocorticoids in the brain influence neuroendocrine function, neuronal excitability, neuronal plasticity, cognition, neurogenesis, and neurodegeneration. While many studies have examined the role of GR in mediating the effects of glucocorticoids in the brain, much less is known about the function of MR in mediating the effects of glucocorticoids in the central nervous system. The goal in this thesis has been to address the role of MR in these processes using both *in vivo* techniques to understand physiological and behavioral consequences of MR action, and *in vitro* techniques to understand cellular consequences of MR action.

### Mineralocorticoid receptor influences HPA axis activity and anxiety-like behavior:

Chapter 2 addressed the physiological and behavioral consequences of MR action using a mouse model that overexpresses MR specifically in forebrain regions. Several important conclusions can be drawn from this chapter. First, the actions of MR in the forebrain can include the negative feedback regulation of HPA axis activity in response to stress. Second, overexpression of MR in the forebrain results in decreased anxiety-related behaviors. Third, overexpression of MR in the forebrain has significant effects on

the basal expression patterns of other genes that are involved in the modulation of both HPA axis activity and anxiety-related behaviors, including decreased GR mRNA expression in region CA1 of the hippocampus and increased expression of 5HT-1a also in region CA1 of the hippocampus.

Over the last few decades, antagonist studies have suggested that the activity of MR is necessary for the maintenance of proper HPA axis activity under basal conditions. While several studies have suggested that the combined activity of both MR and GR may be required to restrain HPA axis activity during stress, this is the first study to suggest that an increase in MR levels alone is sufficient to decrease HPA axis activity in response to stress. The findings of decreased anxiety-related behavior, and of increased expression of 5HT1a in the hippocampus are especially salient with respect to our understanding of MR action because they were not expected. The decreased anxiety-related behavior is interesting as it demonstrates that long-term increases in MR expression can result in a phenotype that directly contrasts with the proposed function of short-term activation of MR. This finding is also interesting because increased expression of GR in the forebrain has the opposite effect of increasing anxiety-like behavior. These data serve to highlight the opposite roles that MR and GR play in anxiety-like behavior and emphasize the importance, at a behavioral level, of a proper balance in MR and GR expression levels. The finding of increased expression of 5HT1a in the hippocampus is interesting in that it also contrasts with *in vitro* data suggesting that MR represses 5HT1a expression at an nGRE found in its promoter, as well as *in vivo* data (adrenalectomy and corticosterone replacement) suggesting that glucocorticoids also repress 5HT1a expression. Even

though this result is unexpected, it does provide a possible mechanism underlying the decrease in anxiety-like behavior.

**Mineralocorticoid receptor protects HT-22 cells from glucocorticoid endangerment:**

There is good evidence that high levels of corticosteroids can endanger neurons, leading to possible deleterious effects on the structure and function of the hippocampus. Studies in chapter three of this thesis have focused on the cellular consequences of glucocorticoid exposure with the goal of determining the roles of MR and GR in this process of glucocorticoid endangerment. For these studies, we chose the hippocampal cell line HT-22. This cell line had previously been shown to express GR, be very sensitive to glutamate toxicity, and to be sensitive to glucocorticoid exacerbation of glutamate toxicity. In subsequent studies we were able to demonstrate that these cells do not express MR, allowing us to make stable clones of HT-22 cells that express MR in addition to GR, and determine in side-by-side experiments with the HT-22/Parent cell line, the ability of glucocorticoids to exacerbate glutamate toxicity. In line with many other studies suggesting a neuroprotective role for MR, we were able to determine that while the parent cell line exhibited an enhancement of glutamate toxicity when pre-exposed to glucocorticoids, clone HT-22/MR16 no longer displayed this phenomenon, suggesting that MR can attenuate glucocorticoid mediated enhancement of glutamate toxicity.

While the exact mechanism underlying this effect remains to be determined, several mechanisms by which MR may play a neuroprotective role in neuronal cells have been proposed. In single CA1 cells, it has been shown that predominant MR activation can alter the ratio NMDA receptor subunit genes (increase NR2A:NR2B) leading to

overall decreased NMDA receptor activity and reduced calcium flux (Nair, Werkman et al. 1998). MR signaling has also been shown to up-regulate anti-apoptotic enzymes Bcl-2 and Bcl-xL and down-regulate p53 expression (Almeida, Conde et al. 2000). Additionally, other studies have shown that the manipulation of MR activity both *in vivo* and *in vitro* can influence cell survival through apoptosis related mechanisms (McCullers and Herman 1998; McCullers and Herman 2001; Crochemore, Lu et al. 2005).

In line with effects on apoptotic mechanisms, we have shown preliminary evidence that when MR and GR are co-activated in HT-22/MR16 cells, the protective effect may come in part through the decreased activation of apoptotic enzymes. It was shown previously that the addition of MR to PC12 cells was able to provide a certain level of neuroprotection to multiple insults including staurosporine and oxygen/glucose deprivation. If the addition of MR to HT-22 cells is capable of altering apoptotic processes, then it would be interesting to test whether HT-22/MR16 cells are more resistant to additional insults such as oxygen/glucose deprivation and staurosporine.

**Altering the ratio of MR to GR significantly changes global transcriptional output relative to GR activation alone:**

There are several reported instances where hyperactivity of the HPA axis is associated with increased susceptibility to neuronal damage or to negative effects on learning and memory as well as mood. In these instances, alterations in the ratio of MR:GR expression in the hippocampus are often found. MR and GR are ligand activated transcription factors that both bind glucocorticoid hormones and regulate the expression patterns of gene networks. Previous research has shown that when MR and GR are

expressed together, their activity on a specific promoter can result in transcriptional regulation that differs from activation of either MR or GR alone. What hasn't been appreciated before is the extent to which variations in the levels of the receptors can alter the transcriptional output on a global scale.

The findings from chapter 4 are important in two ways. First, they suggest that altering the ratio of MR to GR expression in a hippocampal cell line can drastically change the global transcriptional output in response to the same level of hormone. Second, gene ontology analyses of the changed genes in each cell line suggest that when MR and GR are activated together, different classes of genes are regulated compared to when GR alone is activated. These include classes of genes that are involved in cell survival and cell proliferation, providing possible insight into how decreased ratios of MR to GR seen in some pathological states may be functionally significant.

**Future directions:**

There are several features that stand out from this body of work. First, it is obvious from the *in vivo* results that short-term activation or blockade versus long-term increases or decreases in receptor level can often yield opposite results; however, the mechanisms underlying the differences in short-term activation of MR and long-term increases in MR levels remain unclear. Korte, a prominent researcher in the field of glucocorticoid actions in fear and anxiety, suggests that "Corticosteroids do not regulate emotional behavior, rather they induce chemical changes in particular sets of neurons, making certain behavioral outcomes more likely in a certain context, as a result of the strengthening or weakening of particular pathways" (Korte 2001). To extend this

concept, one could suggest that glucocorticoid actions on behavior can perhaps be divided into multiple realms. In a shorter time frame, glucocorticoids can influence cellular responses that, in turn, influence behavior at that moment, or over a period of hours. Over longer periods of exposure to increased glucocorticoid levels, or to long-term alterations in corticosteroid receptor levels, other aspects of cellular properties can also be influenced, such as energy metabolism, that will make that particular set of neurons respond differently perhaps leading to a different behavioral or physiological output. Clearly, if a particular set of neurons that is involved in modulating a particular behavioral output in response to glucocorticoids is now altered in its function, then that set of neurons could now respond differently to glucocorticoids relative to the naïve state. The fact that it is the same hormone that is participating in both aspects of these cellular properties (behavioral modulation versus energy metabolism) suggests that the influence of MR on behavior is context dependent.

In the context of mice that overexpress MR in the forebrain, it may then be interesting to repeat some of the original MR antagonist studies where it was determined that MR blockade reduces anxiety-like behavior. If MR blockade in MRov mice results in a further decrease in anxiety-like behavior, above the already decreased anxiety background, then this could suggest that the immediate actions of MR activation increase anxiety-like behavior, but that long-term elevations in MR levels may serve to influence overall cellular properties in a manner that reduces anxiety under basal conditions. In this case an appropriate question may be: Do neurons that overexpress MR exhibit different basal properties compared to neurons that do not overexpress MR? If so, are these differential properties responsible for altered behavior?

In MRov animals, one can address the basal properties of MR overexpressing neurons at multiple levels. This could include a microarray analysis of laser-captured neurons from specific CA regions or the dentate gyrus. As MR and GR are only expressed in the granule and pyramidal layers of the hippocampus, this would be a powerful way of examining changes in a very region-specific manner and would remove the problems that accompany microarray analysis from hippocampal tissue as a whole, mainly the loss of sub-region specificity, and the dilution of low abundant transcripts amongst the rest of the hippocampal tissue. There are many different kinds of comparisons one could make with this approach. In addition to trying to find genes that are regulated by corticosterone differentially in WT and MRov mice (such as adrenalectomy to low or high corticosterone replacement), it may also be interesting to find genes that are expressed differentially between WT and MRov animals under specific conditions, such as the circadian trough and peak or in a perturbed system such as chronic unpredictable stress.

Another important approach would be to address the cellular properties of hippocampal neurons that overexpress MR. It was recently shown in forebrain specific MR-KO mice that non-genomic, MR mediated actions are required for proper glutamate signaling (Karst, Berger et al. 2005). The overexpression of MR in the hippocampus may specifically alter aspects of cellular properties that could contribute to the behavioral and physiological phenotypes observed in these animals. If this is the case, one very interesting question is whether the MR can transduce glucocorticoid signaling by acting at the membrane. How could this occur? Could MR activation at the membrane

modulate traditional intracellular second messenger pathways such as the MAPK and PI3K pathways?

Research that is especially pertinent to our understanding of the interactions between HPA axis regulation and mood disorders includes studies that address chronic stress. Previous studies have shown that conditions that lead to chronic elevations in glucocorticoids, such as chronic unpredictable stress (CUS), can have several effects on the structure and function of the hippocampus including increased susceptibility of neurons to toxic insults, decreased neurogenesis, increased anxiety-like behavior, and decreased learning and memory. These studies suggest that there may be alterations in the ratios of MR to GR in the hippocampus and that these changes may contribute to hippocampal structure and function. If increased levels of MR in the hippocampus lead to decreased anxiety under basal conditions, it would be very interesting to determine the effects of increased MR on all these aspects of hippocampal structure and function under conditions of chronic stress.

**MR and GR balance in glucocorticoid signaling:**

The second prominent feature that arises from both the *in vivo* and *in vitro* research in this thesis, as well as similar work from other labs, is the idea that a proper ratio of MR to GR in the hippocampus is important for multiple aspects of appropriate physiological, behavioral and cell survival processes. Considering the importance of the combined roles of MR and GR signaling in the same cell, it is surprising how little is actually known concerning the mechanisms underlying heterodimer versus homodimer action. For example, we know that GR can either repress or activate AP1 activity when it

is composed of cJUN/cFOS heterodimers or cJUN/cJUN homodimers, respectively, at either a cGRE or juxtaposed simple GRE and AP1 binding site (Diamond, Miner et al. 1990; Pearce, Matsui et al. 1998). It has been shown that while MR can bind to this same site with equal affinity, it has no effect on AP1 activity (Pearce and Yamamoto 1993). However, nothing is known regarding the effects of MR/GR heterodimers on cGRE activity.

Liu and colleagues reported that the co-expression of MR and GR was capable of inhibiting the levels of induction of reporter gene activity as compared to GR activation alone (Liu, Wang et al, 1995). This study used a reporter gene containing 3 tandem GREs in the promoter, and they propose that this MR-mediated inhibition of GR activity may be due to an MR-mediated disruption of GR self-synergy at the GRE multimer. However, the reporter gene used in our studies also contained a multimer of 5 GREs in the promoter and in contrast to the effects observed by Liu and colleagues, we found greatly increased levels of reporter gene activity when MR was present at different levels along with GR, suggesting that MR does not restrict GR self-synergy in all contexts. Additionally, we know that MR and GR can interact with distinct sets of coregulators, but we know nothing of the interactions of MR:GR heterodimers with coregulators. On a given promoter, are the same coregulators recruited by a heterodimer as the respective homodimers? Are receptor specific coregulators still recruited to a heterodimer? It may be possible to address some of these questions with the MR and GR point mutants developed by Liu and colleagues (Liu, Wang et al. 1995). These mutants can only heterodimerize, thus allowing one to determine if an MR specific coregulator (FAF-1) can still be recruited relative to MR:MR homodimers.

As the N-terminal regions of MR and GR are responsible for many of the interactions they have with other coregulators, and they share only 15% amino acid identity, one could hypothesize that heterodimer action is largely mediated by differential interactions of heterodimers with coregulators. To test this hypothesis, it would be interesting to swap the N-terminal domain of MR with the N-terminal domain of GR. After making stable clones of HT-22 cells with this chimera, one could test if it is largely the same or different genes that are being regulated relative to the HT-22/Parent cell line at the same dose of corticosterone.

Beyond the use of the MRov mice used in this project, it would also be interesting to generate other mouse models that could further test the idea that the ratio of MR to GR is important in hippocampal function. While mice that are lacking MR in the forebrain do not appear to have any disturbances in anxiety-related behavior, it could be fruitful to develop mice that have decreased levels of MR rather than a complete loss of MR. The decreased ratio of MR to GR in the hippocampus in transgenic mice may more closely resemble decreased ratios of MR to GR that accompanies chronic stress or aging. Additionally, it would be very interesting to develop forebrain specific knock-in mice with a dimerization defective MR, or even to generate knock-in animals that are only capable of generating MR/GR heterodimers using the salt-bridge mutants that were developed by (Liu, Wang et al. 1995). These mice could be used to study the physiological and behavioral effects of altering MR homodimer and MR/GR heterodimer signaling.

**Conclusions and perspectives:**

Diseases of affect produce a very large burden, both emotionally and financially on individuals, families, and ultimately the community and overall economy. Major depression and anxiety disorders are extremely complex diseases. It may very well be true that, in a manner analogous to cancer, the overall expression of the disease state in any one individual might be due to many different underlying causes, both genetic and environmental. Not every depressed patient exhibits hypersecretion of glucocorticoids and not every depressed patient is treatable with the same, or even any, antidepressants, suggesting that we are still far from a complete understanding of the mechanisms leading to anxiety and depression.

Research that addresses novel therapeutic strategies for major depressive disorders suggests that one common theme in depression may be disturbances in neural plasticity pathways. Glucocorticoids have powerful effects on neural plasticity, ranging from modulating LTP in hippocampal neurons to affecting neuronal survival in response to toxic insults, to facilitating glutamate transmission, to regulating the expression of neurotrophic factors (Carlson, Singh et al. 2006). Dysregualtion of the HPA axis is often seen depression, as well as other mood disorders, suggesting that variations in glucocorticoid levels, or levels of their receptors, may contribute to the underlying causes of some mood disorders.

While there are still many questions regarding the underlying mechanisms by which MR and GR, both individually and together, mediate glucocorticoid effects, the research in this thesis extends our understanding of the role of MR in mediating glucocorticoid effects in the central nervous system by demonstrating roles for MR that

are both similar to and distinct from the roles of GR. *In vivo,* the overexpression of MR in the forebrain can suppress HPA axis activity during stress, in a manner that is similar to the proposed role for GR, and decrease anxiety-like behavior, in a manner that is distinct from the proposed role of GR. *In vitro,* research in this thesis also demonstrates that the co-activation of MR and GR in a hippocampal cell line can attenuate the glucocorticoid endangerment effect mediated by activation of GR alone, suggesting distinct roles for GR and MR in mediating effects on neuro-endangerment versus neuroprotection. Finally, research presented in this thesis demonstrates that when MR and GR are co-activated in a hippocampal cell line, entirely different sets of genes may be regulated compared to when GR alone is activated, suggesting how altered ratios of MR:GR in the same cell may significantly affect glucocorticoid regulation at the transcriptome level.

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